UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

The Sabah Rhino Breeding Programme: reproductive management of the critically endangered Sumatran rhinoceros of Borneo (*Dicerorhinus sumatrensis harrissoni*) as conducted by the IZW-Berlin between 2005 and 2015.

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DECLARAÇÃO RELATIVA ÀS CONDIÇÕES DE REPRODUÇÃO DA TESE

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Orientadores: Prof. Dr. Thomas Bernd Hildebrandt

Dr. Sandra de Oliveira Tavares de Sousa Jesus

Ano de conclusão: 2017

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Assinatura:

Rafaela Fiúza

To everyone out there fighting for rhinos.

"As long as prevailing resistance remains from the relevant governments, IUCN, and the big NGOs, then the species will go extinct, and those institutions, not poachers or palm oil producers, will have to shoulder most of the ensuing blame."

Dr. John Payne, Executive Director of the Borneo Rhino Alliance.

"Insanity is doing the same thing over and over again, but expecting different results."

Unknown origin. Commonly attributed to Einstein.

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To Thomas Hildebrandt, for believing in me.

To Thomas Hildebrandt, Frank Göritz, Robert Hermes, Johanna Painer, Susanne Holtze, Sónia Fontes, Julia Bohner and Nga Nguyen, for everything you have taught me.

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A Henrique Fiuza e Maria Fiuza, por me deixarem sonhar. A Catarina Fiuza, por partilhar o amor pelos animais, e esta vida, comigo. À Pipoca, irmã de outra espécie, por unir a nossa família. A Celeste Barbosa e Rosinda Ferreira, por nos passarem este amor pelos animais.

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À nossa FMV, Veterinária Cilindra!

Para sempre, Vossa.

Forever, Yours.

To the Leibniz Institute for Zoo and Wildlife Research (IZW-Berlin), the Borneo Rhino Alliance (BORA), the Sabah Wildlife Department, the Yayasan Sime Darby Foundation and the Bundesministerium für Bildung und Forshung for making the Sabah Rhino Project possible.

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ABSTRACT

The Sabah Rhino Breeding Programme: reproductive management of the critically endangered Sumatran rhinoceros of Borneo (*Dicerorhinus sumatrensis harrissoni*) as conducted by the IZW-Berlin between 2005 and 2015.

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is on the verge of extinction. Once found throughout Southeast Asia, it stands now with less than 100 individuals scattered mainly in three national parks in Sumatra. The IZW-Berlin has been collaborating with BORA/SWD through the use of advanced imaging and assisted reproduction techniques in wild-caught Bornean rhinoceroses (*D. s. harrissoni*) held at the BRS. Ultrasonographic examinations and reproductive procedures conducted between 2005 and 2015 in 2 male and 3 female rhinoceroses were retrospectively analysed in order to infer on reproductive condition and its evolution, outcome of procedures and impact of interventions. Furthermore, the study aimed at developing a detailed description of ultrasonographic findings and identify evidence to further elucidate on the estrous cycle of the species. A total of 17 working visits were included in the descriptive analysis with 56 reproductive assessments by ultrasonography, 8 semen collections by electroejaculation, 10 hormonal treatments, 1 artificial insemination, 5 oocyte collections, 3 intracytoplasmic sperm injections, 4 techniques for the removal of endometrial cysts and 1 hydrosalpinx aspiration. The detailed description of findings provides new technical information on numerous anatomical structures of both males and females, and constitutes the first report of various procedures in the species. Notably, several hypotheses are considered and put forward for future investigation. Results revealed that no animal suffered negative consequences from the repeated and sometimes invasive interventions. While the older female was found to have entered early reproductive senescence, the two young females were reproductively active but showed a severely impaired reproductive tract, namely affected by endometrial cystic hyperplasia and uterine leiomyomas. These findings were associated to the phenomenon of asymmetric reproductive ageing and a presumptive relationship with lack of breeding and low densities in the wild is reported. Ultrasonography further revealed interesting and novel findings on estrous cyclicity, which in combination with observed pathological changes may explain the lack of success of most reported procedures. In regard to the males, semen collections were successful but semen quality proved to be very low, and hypotheses of this being related to husbandry issues and/or pathological changes are reported.

Keywords: rhinoceros, Sumatran, reproduction, cycle, pathology, cyst, leiomyoma, semen.

RESUMO

Programa de Reprodução do Rinoceronte de Sabah: maneio reprodutivo do rinoceronte-de-Sumatra do Bornéu (*Dicerorhinus sumatrensis harrissoni*) criticamente ameaçado de extinção, como conduzido pelo IZW-Berlim entre 2005 e 2015.

O rinoceronte-de-Sumatra (*Dicerorhinus sumatrensis*) encontra-se à beira da extinção. Outrora presente em todo o sudeste asiático, está agora reduzido a menos de 100 indivíduos separados por três parques nacionais na Sumatra. O IZW-Berlim tem colaborado com a BORA/SWD através do uso de técnicas avançadas de imagem e reprodução assistida nos rinocerontes do Bornéu (*D. s. harrissoni*) capturados do meio selvagem e mantidos no BRS. Exames ultrassonográficos e procedimentos reprodutivos realizados entre 2005 e 2015 em 2 machos e 3 fêmeas foram analisados retrospetivamente para inferir sobre condição reprodutiva e sua evolução, resultado dos procedimentos e impacto das intervenções. Adicionalmente, procurou-se desenvolver uma descrição detalhada dos achados ecográficos e identificar evidências que ajudem a compreender o ciclo éstrico da espécie. Um total de 17 visitas foram incluídas na análise descritiva com 56 avaliações reprodutivas por ultrassonografia, 8 recolhas de sémen por electroejaculação, 10 tratamentos hormonais, 1 inseminação artificial, 5 recolhas de oócitos, 3 injeções intracitoplásmicas de espermatozóides, 4 técnicas para a remoção de quistos endometriais e 1 aspiração de hidrossalpinge. A descrição detalhada dos achados fornece nova informação técnica sobre numerosas estruturas anatómicas de machos e fêmeas, e constitui o primeiro registo de diversos procedimentos na espécie. Notavelmente, várias hipóteses são avançadas para investigação futura. Os resultados revelaram que nenhum animal sofreu consequências negativas das intervenções repetidas e por vezes invasivas. Enquanto a fêmea mais velha se encontrava em senescência reprodutiva precoce, as duas fêmeas mais jovens exibiam atividade reprodutiva mas também um aparelho reprodutivo extremamente afetado por hiperplasia quística do endométrio e leiomiomas uterinos. Estes achados foram associados ao fenómeno do envelhecimento reprodutivo assimétrico, e uma relação presuntiva com ausência de atividade reprodutiva e baixas densidades em meio selvagem é reportada. A ultrassonografia revelou ainda novos achados em relação ao ciclo éstrico, que em conjugação com as alterações patológicas observadas poderão explicar a falta de sucesso da maioria dos procedimentos relatados. Em relação aos machos, recolhas de sémen foram realizadas com sucesso mas revelaram uma qualidade muito baixa, e hipóteses desse facto estar relacionado com problemas de maneio e/ou alterações patológicas são reportadas.

Palavras-chave: rinoceronte, Sumatra, reprodução, ciclo, patologia, quisto, leiomioma, sémen.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABI	Agro-Biotechnology Institute
AI	artificial insemination
AV	artificial vagina
BC	Berliner Cryomedium
BORA	Borneo Rhino Alliance
BRS	Borneo Rhino Sanctuary
CH	*corpus hemorrhagicum*
CL	*corpus luteum*
cm	centimetre
CMA	chlormadinone acetate
COC	*cumulus*-oocyte complex
Diplo.	diplomate
DMSO	dimethylsulfoxide
Dr.	doctor
eCG	equine chorionic gonadotropin
eFSH	equine follicle stimulating hormone
e.g.	for example (*exempli gratia*)
EJ	electroejaculation
fig.	figure
FSH	follicle stimulating hormone
GnRF	gonadotropin releasing factor
GnRH	gonadotropin releasing hormone
GVBD	germinal vesicle breakdown
h	hour
ha	hectare
HAF	hemorrhagic anovulatory follicle
hCG	human chorionic gonadotropin
hFSH	human follicle stimulating hormone
ICSI	intracytoplasmic sperm injection
iPSC	induced pluripotent stem cells
IRF	International Rhino Foundation
IU	international unit
IUCN	International Union for the Conservation of Nature
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IZW	Leibniz Institute for Zoo and Wildlife Research
kg	kilogram
km^2	square kilometre
L	litre
LH	luteinizing hormone
LKWP	Lok Kawi Wildlife Park
m	metre
mA	milliampere
Me_2SO	dimethylsulfoxide
mg	milligram
MHz	megahertz
min	minute
ml	millilitre
mm	millimetre
MoF	Ministry of Forestry of the Republic of Indonesia

n	sample size
ng	nanogram
NP	national park
OPU	ovum pick-up
PC	post-coital
PCR	polymerase chain reaction
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PM	penile massage
Prof.	Professor
RBC	Rhino Breeding Centre
RM	transrectal massage of the accessory sex glands and pelvic urethra
SD	standard deviation
SEM	standard error of the mean
spz	spermatozoon
SSP	Species Survival Plan
SWD	Sabah Wildlife Department
TCM	tissue culture medium
TR	transrectal
TV	transvaginal
TWR	Tabin Wildlife Reserve
UK	United Kingdom
USA	United States of America
V	volt
v/v	volume/volume
vs	versus
WAZA	World Association of Zoos and Aquariums
WWF	World Wildlife Fund
µg	microgram
µl	microlitre
/	fraction (with numbers)
%	percentage
º	degree
ºC	degree Celsius

INTERNSHIP REPORT

As part of the Integrated Master in Veterinary Medicine from the Faculty of Veterinary Medicine of the University of Lisbon (FMV-ULisboa), the author undertook a curricular internship at the Leibniz Institute for Zoo and Wildlife Research (IZW) in Berlin, Germany. The internship was conducted in the department of Reproduction Management under the supervision of the department director, Prof. Dr. Thomas B. Hildebrandt. Initial length of the internship was six months, but once given the opportunity it was extended to one full year, amounting to well over 1000 hours.

The Leibniz Institute for Zoo and Wildlife Research is an interdisciplinary research institute whose mission is to conduct evolutionary wildlife research in order to develop the scientific basis for novel approaches to wildlife conservation. Their vision is focused on understanding and improving the adaptability of wildlife species, being adaptability the term used to describe the evolutionary (genetic and phenotypic) potential to respond to natural or anthropogenic environmental changes, including both the resistance (the extent to which wildlife is affected in the short term by environmental change) and the resilience (the speed at which a population recovers after a challenge). The department of Reproduction Management is focused on reproductive strategies and the emergence of human-induced reproductive disorders in free-living and captive wildlife. For that they apply modern imaging technologies such as ultrasonography and computed tomography, and develop new techniques for assisted reproduction in order to optimize the reproductive management of endangered species.

During the internship most of the opportunities came with assisting the various researchers of the department in their practical endeavours. The student helped with preparations, anesthesia of wildlife, clinical procedures, ultrasonographic assessments (reproductive and others), assisted reproduction techniques (semen collections, artificial inseminations, oocyte collections), amongst others. The intern was often responsible for developing reports on the clinical procedures, anesthesia and ultrasonographic findings. Main wildlife species with which the student had the opportunity to work were elephants (Asiatic and African), rhinoceroses (southern and northern White, Indian, Black and Sumatran), European brown bear and Asiatic lion. Other species with which the intern had contact and worked at least once were Malayan sun bear, striped and spotted hyenas, grey wolf, bobcat, wild boar, pigmy hippopotamus, and some reptiles such as tuataras. Activities were conducted in zoos and wildlife sanctuaries in Germany, Czech Republic, Poland, the Netherlands, Malaysia and Singapore. Apart from the specific work of the department, the intern also had the opportunity to assist with the procedures conducted on animal colonies held for research purposes at the institute and respective field station, namely naked mole-rats, European roe deer, mountain hares and wild guinea pigs.

During the second half of the internship more responsibilities were bestowed on the student. On a certain number of days, the intern was responsible for managing the naked mole-rat colonies, including feeding, cleaning, record keeping and processing of new litters (counting, registration and collection of biological samples). The development of laboratory work was another new responsibility, namely molecular sexing of the new-born naked mole-rats through a multiplex polymerase chain reaction (PCR) assay and agarose gel electrophoresis. Some administrative tasks were also performed such as planning of trips and ordering of supplies.

On a final topic, during the length of the internship the student had the opportunity to be present in a number of lectures, including a short course on the "Theory of Science" by Prof. Dr. Heribert Hofer, and the IZW Seminar held once every four years. The student also developed two abstracts during the internship and a third one after its conclusion, which were presented in the form of scientific posters at the International Conference on Diseases of Zoo and Wild Animals 2015 in Barcelona and the 10th International Conference on Behaviour, Physiology and Genetics of Wildlife in Berlin; and as an oral presentation at the 12th Conference of the European Wildlife Disease Association also in Berlin in 2016 (see appendix).

In regard to the scientific work developed for the present thesis, a very large amount of time was spent analysing ultrasound examinations of Sumatran rhinoceroses. For that a short training on ultrasonography was conducted with Prof. Dr. Thomas Hildebrandt and Dr. Robert Hermes, and a lot of personal effort and persistency was necessary. On the same subject an important note should be taken: the department and especially Prof. Dr. Thomas Hildebrandt gave the intern the amazing opportunity to travel with the team to Sabah (Malaysian Borneo) and assist with the work conducted on the Sumatran rhinoceroses of the study. The trip took place in April 2015 and was the last trip included in the present study. The intern had the opportunity to observe and assist with anesthesia, reproductive assessments of male and females through ultrasonography, semen collection by electroejaculation, follicle aspiration for oocyte collection, and aspiration of an hydrosalpinx.

The student believes the internship conducted was beneficial on several levels, both professionally and personally. Most importantly it was an incredible opportunity to witness and experience the unique work conducted by this department and the leading scientists that compose it. In terms of scientific knowledge and forward thinking it was truly inspiring, and it made the student work harder and aim at being and doing the best possible. Although clinical training was not the strongest point, the student came out stronger in several different areas, and is truly thankful for that.

INTRODUCTION

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is on the verge of extinction. Once found throughout Southeast Asia, it stands now with less than 100 individuals scattered mainly in three national parks in Sumatra. In Sabah (Malaysian Borneo), as well as in Peninsular Malaysia, the Sumatran rhinoceros is now considered to be extinct. In 1984 it was agreed that in addition to the protection of wild individuals, it was time to establish a captive breeding programme. In the three decades that followed, forty-five Sumatran rhinoceroses were captured from the wild, unexpectedly resulting in only two breeding pairs. In 2016 only ten individuals survived in captivity: three (one male and two females) at the Borneo Rhino Sanctuary, in Sabah; and seven (two adult males, three adult females and two juveniles) at the Sumatran Rhino Sanctuary, in Sumatra.

For many years the Leibniz Institute for Zoo and Wildlife Research (IZW-Berlin) has been collaborating with the Borneo Rhino Alliance (BORA, previously known as SOS Rhino Borneo) and the Sabah Wildlife Department (SWD) through the search and monitoring of wild populations, and through the use of advanced imaging and assisted reproduction techniques on the wild-caught Sabah rhinoceroses (*Dicerorhinus sumatrensis harrisonni*).

The present research project focuses on data obtained during the interventions of the IZW scientists on the wild-caught individuals held in captivity in Sabah between 2005 and 2015. All ultrasonographic examinations were retrospectively analysed by the author, and information on all procedures conducted on said rhinoceroses was also collected and analysed.

The overall aim of the present study was to analyse the evolution of the programme and help the IZW team understand where to focus in the near future, in order to improve results and achieve the final goal of helping saving the species. Specific objectives were to 1) analyse the evolution of each animal in terms of reproductive health using ultrasonographic findings and clinical reports; 2) evaluate the procedures conducted through time and their outcome, and infer on their importance; 3) assess the impact of the work conducted by the IZW team on the individuals under study; 4) develop a detailed description of the ultrasonographic characteristics of the reproductive tract of Sumatran rhinoceroses; 5) find evidence to corroborate or discredit the existing theories regarding the estrous cycle of the species.

AN UPDATED REVIEW OF LITERATURE

The rhinoceros, often abbreviated as “rhino”, is a large mammal belonging to the family Rhinocerotidae, one of the three surviving families of the order Perissodactyla (odd-toed ungulates). There are five extant species in the family Rhinocerotidae, divided in four genera, and present naturally in two continents: the White and the Black rhinoceroses in Africa, and the Indian, the Javan and the Sumatran rhinoceroses in Asia.

The White rhinoceros (or less commonly the Square-lipped rhinoceros), *Ceratotherium simum*, is classified by the IUCN (International Union for the Conservation of Nature) Red List of Threatened Species as “Near threatened” due to the increasing importance of poaching (Emslie, 2012a), in spite of having the largest numbers of all rhino species with approximately 21 000 wild individuals in 2016 (International Rhino Foundation [IRF], 2016b). Two subspecies of White rhinoceros are recognized: the Southern White rhinoceros, *C. s. simum*; and the Northern White rhinoceros, *C. s. cottoni*, with only three known individuals alive in 2016.

The Black rhinoceros (or less frequently the Hook-lipped rhinoceros), *Diceros bicornis*, is listed as “Critically endangered” by the IUCN Red List of Threatened Species, on account of an estimated decline of 97,6% since 1960 to just over 2000 individuals in 1993 (Emslie, 2012b). By 2016 it is estimated that 5000 to 5500 Black rhinoceroses survive (IRF, 2016b), divided in three extant subspecies or ecotypes occupying different geographical areas in Africa: *D. b. bicornis*, *D. b. michaeli* e *D. b. minor* (Emslie, 2012b).

In Asia two of the rhinoceros species share a genus: the Indian rhinoceros or Greater one-horned rhinoceros, *Rhinoceros unicornis*; and the Javan rhinoceros or Lesser one-horned rhinoceros, *Rhinoceros sondaicus*. While the Indian rhinoceros is listed as only “Vulnerable” by the IUCN Red List of Threatened Species (Talukdar *et al.*, 2008), having a little over 3300 wild individuals in 2016 (IRF, 2016b); the Javan rhinoceros is classified as “Critically endangered” (van Strien *et al.*, 2008b), with no more than 63 individuals left in one population on the island of Java, Indonesia (IRF, 2016b).

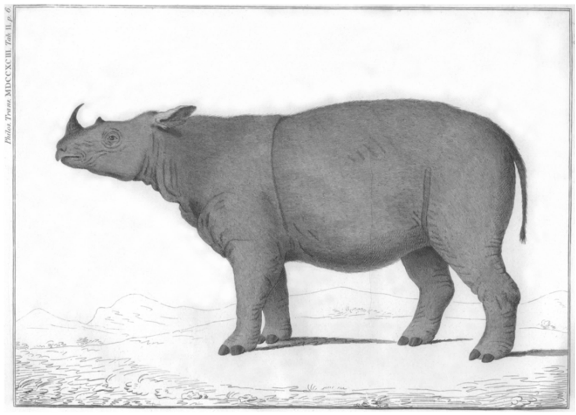
1. The Sumatran rhinoceros

The Sumatran rhinoceros, *Dicerorhinus sumatrensis,* is since 1996 listed as “Critically endangered” by the IUCN Red List of Threatened Species, after ten years under the “Endangered” classification (van Strien *et al.*, 2008a). Also known as Asiatic double-horned rhinoceros or Hairy rhinoceros, the Sumatran rhinoceros stands today with less than 100 individuals left in the wild, and is considered the most threatened of all rhinoceros species due to the fast and sustained decline in its numbers (IRF, 2016b).

1.1. Taxonomy

The first description of a double-horned rhinoceros in Asia was made by William Bell in 1793 (van Strien, 1974). The animal had been shot in Sumatra the day before of the dissection (fig. 1), and besides having two horns, his skin was covered in short black hair and did not have the appearance of armour observed in the single-horned rhinoceros (Bell, 1793). After a long period of disagreement, the name *Dicerorhinus sumatrensis* prevailed (van Strien, 1977), and several subspecies where described of which three are acknowledged since 1967 (Groves, 1967):

Figure 1: Illustration made by William Bell in 1793.



- *D. s. sumatrensis*: described by Fischer in 1814; present in Sumatra and Peninsular Malaysia; large in size, with medium to small teeth and a narrow and low occiput.
- *D. s. lasiotis*: described by Buckland in 1872, found from northern Burma (Myanmar) into Assam and east Pakistan; with denser and longer hair, a larger size, very large teeth and a broad and high occiput (extinct).
- *D. s. harrissoni*: designated only in 1965 by Groves; present in Borneo; small in size, with small teeth and a narrow occiput but proportionally high and forwardly inclined.

1.2. General appearance, ecology and behaviour

The Sumatran rhinoceros is the smallest of all rhinoceros species, measuring approximately 1,35 m in height to the shoulder and 2,50 m in length from snout to tail base. Difference between male and female is not reported as significant. Two horns are present, one smaller above the eyes and one much larger located above the nose. The skin is generally smooth, with one large fold caudal to the forelimbs and one cranial to the hindlimbs. Colour is a mixture of brown, grey and pink. The presence of thick hair covering the body is one of the most striking features, although extent and density vary among animals and are stronger in the young. On the mouth, the Sumatran rhinoceros presents two lower tusk-like teeth (incisors or canines depending on the authors) which are used to fight (van Strien, 1974; Groves & Kurt, 1972).

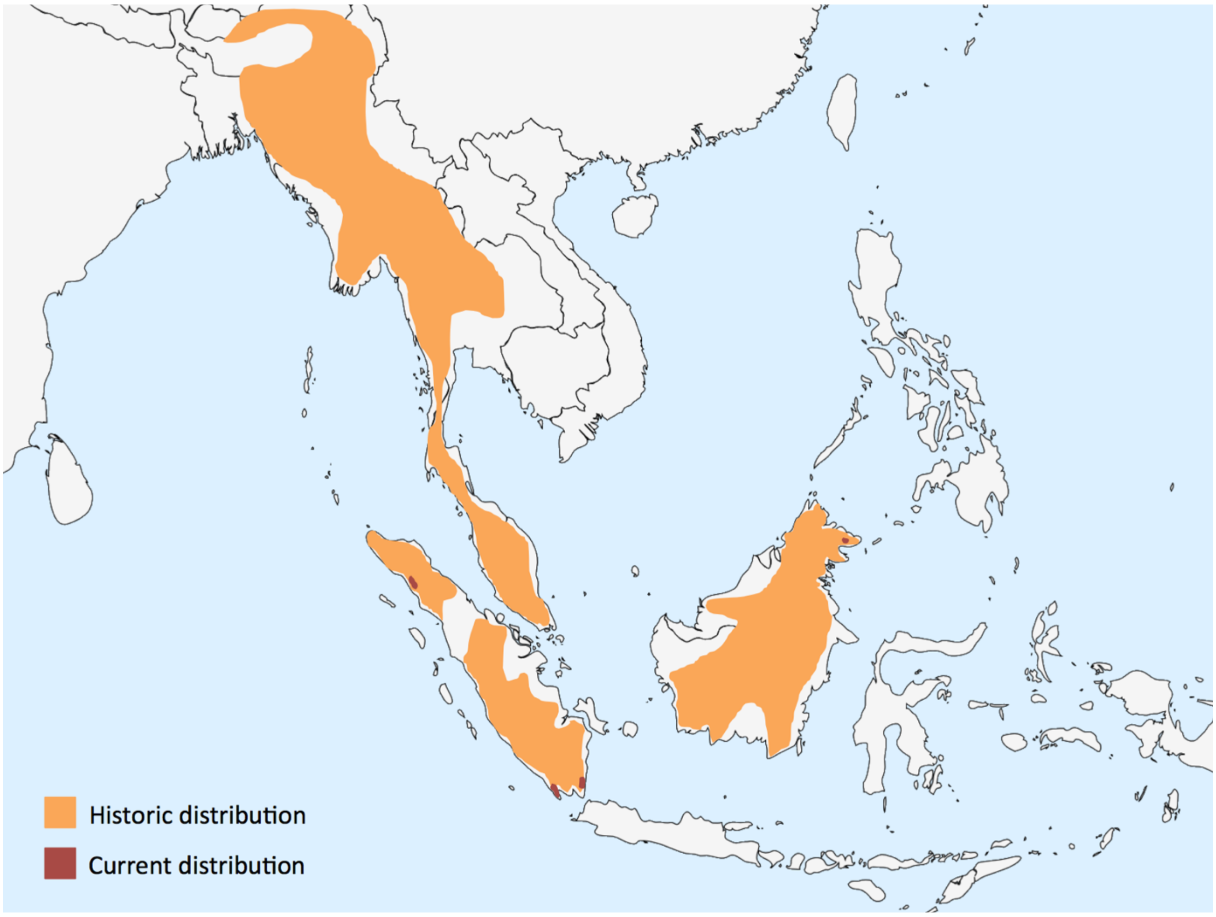
The Sumatran rhinoceros inhabits dense tropical rainforests preferably in the mountains (sometimes over 1500 m in altitude) although they also go down to lower forests, grasslands and man-made landscapes where they can feed on cultivated plants. Sumatran rhinoceroses are mostly solitary animals, sporadically seen in small associations between male and female (sometimes with calf) or up to four adults. Home ranges vary between approximately 10 km^2

(non-breeding females and sub-adults) and 30 km^2 (adult males), possibly overlapping in the best feeding areas and salt licks, which they visit to balance the mineral composition of food. The Sumatran rhinoceros is a typical browser, feeding on leaves, twigs, fruits and bark of a large number of plants; and is mostly nocturnal, feeding before dawn and after dusk, and spending the day in mud wallows (van Strien, 1974; van Strien, 1985; Groves & Kurt, 1972).

1.3. Population density and distribution

The original range of the Sumatran rhinoceros was vast in the second half of the 1800's (fig. 2), reaching from the Himalayas through Myanmar, Thailand and Peninsular Malaysia, down to the islands of Sumatra and Borneo. Even when widely distributed, the Sumatran rhinoceros was seldom described as plentiful (Foose & van Strien, 1997). By the turn of the 20th century the species was starting to lack on the continent but was still considered common on the islands (van Strien, 1974). While the rhinoceros soon disappeared from its northern range, in Peninsular Malaysia estimations varied between 10 and 75 individuals until 1995, when the IUCN reported 41 known and 78 estimated rhinoceroses (Foose & van Strien, 1997). Ten years later official records revealed only 15 individuals, and by 2013 the species was considered locally extinct (Ahmad, Payne and Zahari, 2013). On the island of Borneo, the Sumatran rhinoceros was declared extinct from the Malaysian state of Sarawak in 1968 (van Strien, 1974), but survived until recent times on the other Malaysian state, Sabah. While Payne reported an estimate of only 10 individuals in 1990, the IUCN claimed there were 30 known and 70 estimated rhinoceroses in Sabah in 1995 (Foose & van Strien, 1997). Until 2009 these estimates were maintained and supported (Talukdar *et al.*, 2009) but after that time, and despite extensive surveys, there were no more signs of wild rhinoceroses in Sabah except for two females which were captured in 2011 and 2014. Havmøller *et al.* stated in 2015 that it is safe to assume the species is locally extinct in Sabah, and finally extinct in all of Malaysia. On the Indonesian part of Borneo, Kalimantan, the Sumatran rhinoceros soon disappeared and by 1995 was declared locally extinct (Foose & van Strien, 1997). In 2013 however, camera-trap footage of a rhinoceros was obtained, and in March 2016 a female was captured and news of 15 rhinoceroses being found in that area became public (World Wildlife Fund [WWF], 2013; WWF, 2016). The 4-year-old female named Najaq was placed under governmental care and died tragically only three weeks after her capture (Woollaston, 2016). On the island of Sumatra (Indonesia), the IUCN and other organizations estimated in 1993 the existence of 215 to 319 rhinoceroses (Soemarna *et al.,* 1994), but in 1995 found only proof of 39 animals and reported the estimate of 150 individuals (Foose & van Strien, 1997). Official records of Indonesia claimed the island held 147 to 220 rhinoceroses in 2004, and revealed that one of the largest populations (believed to comprise 80 to 100 animals in 1993) had disappeared within ten years (Ministry of Forestry of the Republic of Indonesia [MoF], 2007). In 2014 Nardelli stated there were probably only 75 rhinoceroses in all of Sumatra, and Pusparini reported similar results: approximately 26 (12 to 61) in Gunung Leuser NP, 31 (19 to 66) in Bukit Barisan Selatan NP and 27 (14 to 50) in Way Kambas NP. Although real numbers are not known, a consensus exists since 2013 that less than 100 Sumatran rhinoceroses survive in the wild (fig. 2; IUCN, 2013; IRF, 2016b).

Figure 2: Compound map showing both the approximate historic distribution and current distribution of the critically endangered Sumatran rhinoceros. Distribution information referenced on chapter 1.3. (page 4) and base map retrieved from http://wiki.alternatehistory.com/lib/exe/fetch.php?media=seasia1000base.png



1.4. Threats

Hunting is historically seen as the most important threat to the Sumatran rhinoceros, and the near extinction of both Sumatran and Javan rhinoceroses in modern times is largely attributed to the trade during Tang dynasty up to the year 900 (Rabinowitz, 1995). Nowadays poaching in no longer considered the most significant threat, and the last recorded poaching event of a Sumatran rhinoceros happened in Way Kambas NP (Sumatra) in 2007 (Havmøller *et al.*, 2015). The second most significant threat has been historically reported as habitat loss (Rabinowitz, 1995). During the 20th century Sumatran rhinoceroses were separated and isolated in small populations due to extensive habitat destruction from logging, forest clearance for agricultural development and human settlement (Flynn & Abdullah, 1984). Nowadays the species is confined to protected lowland forests or high-altitude forests largely inaccessible for conversion, which means that habitat loss is no longer considered the most significant threat to its survival (Zafir *et al.*, 2011). Scientists believe the biggest threat to Sumatran rhinoceros conservation at this point is the low population size in itself (Zafir *et al.*, 2011). As described by Allee in 1949, there is a positive correlation between population size or density and mean individual fitness, meaning that with low numbers comes a decline in breeding success (“the Allee effect"; Ahmad *et al.*, 2013). As a result of this we have seen that: numbers of Sumatran rhinoceros have been declining for several decades; populations are isolated and densities are very low presumably contributing to inbreeding and a narrow genetic base; there have been no sightings or reports of juveniles for decades; a skewed sex ratio was observed both in Peninsular Malaysia and Sabah; and reproductive tract pathology is common among wild females. Most authors believe this is the reason for decline of the Sumatran rhinoceros in the second half of the 20th century, even in places with suitable habitat and zero poaching (Ahmad *et al.*, 2013).

1.5. Past conservation efforts

A formal process for implementing an international conservation plan for the Sumatran rhinoceros was initiated in October 1984 with a meeting attended by representatives from Indonesia, Malaysia, Sabah, the UK and USA breeding groups, the IUCN and further researchers familiar with the species. There was a general agreement the Sumatran rhinoceros was greatly endangered, and that intervention was necessary for the species to survive. It was decided that primary support would be given to viable populations in large areas of protected native habitat, especially in the form of anti-poaching units; an educational programme would be created to enhance public awareness and support for the species; and that a captive breeding programme to preserve the genetic diversity of the species would be developed in the countries of origin along with Europe and the USA using animals without hope of long term survival in the wild (Maguire, Seal & Brussard, 1987). Between 1984 and 1996 a total of 40 Sumatran rhinoceroses were captured from the wild and placed into captivity (Foose & van Strien, 1997). By 2015 five more rhinoceroses had been removed from the wild, but 39 of the original 40 had died. The original programme resulted in only one breeding pair in Cincinnati Zoo (USA) which produced three calves. One of those second generation rhinoceroses went back to Indonesia where he successfully bred with one female, resulting so far in two calves. In 2016 a total of eight adult and two young rhinoceroses survive in captivity (six wild-caught and four captive-born) at the Sumatran Rhino Sanctuary in Sumatra and the Borneo Rhino Sanctuary in Sabah (Ahmad *et al.*, 2013; Ellis & Candra, 2012; IRF, 2016a; BORA, 2015).

2. Reproductive anatomy of the female rhinoceros

The first published report on the visceral anatomy of the Sumatran rhinoceros was developed by Garrod in 1873, where he claims to having determined “many points in its anatomy previously unknown” such as the characteristics of the female reproductive tract. As in other rhinoceros species, the Sumatran rhinoceros external genitalia consists of an elongated vertical vulva with outer and inner symmetrical labia. While the dorsal commissure is rounded, the ventral commissure forms a convex structure that protrudes caudoventrally. The outer vulval labia are thick and wrinkled, and in the Sumatran rhinoceros covered with coarse hairs. Measurements taken from six adult female Sumatran rhinoceroses resulted in an average vulval dimension of 8,1 by 6,9 cm. The clitoris is located in the clitoral fossa 2,0 to 3,0 cm above the ventral commissure of the vulva. It is short and flattened dorsoventrally measuring 1,0 to 1,5 cm (Zahari, 1995; Zahari, Rosnina, Wahid & Jainudeen, 2002). The two most distinct traits of the reproductive anatomy of female rhinoceros are the hymeneal membrane and the cervix (Hermes & Hildebrandt, 2012). A hymeneal membrane separates vestibule and vagina in nulliparous females, cranially to the external urethral orifice. In young females the hymen is microperforated or cribriform, having only small openings of 0,1 to 0,5 cm. It then ruptures during the first mating event, and only small flaps of tissue remain (Schaffer, Foley, Gill & Pope, 2001a; Hermes *et al.*, 2006a; Hermes & Hildebrandt, 2012). In older females in which intromission has never occurred, the hymen becomes more fibrous and therefore more difficult to rupture, despite dilated hymeneal orifices (up to 3,0 cm). The persistent hymen is associated to reproductive failure, as it indicates lack of previous mating and also works as a mechanical breeding barrier. Surprisingly, the prevalence of persistent hymen proved to be higher than 90% among adult nulliparous White rhinoceroses belonging to the European, North American and Australian Species Survival Plan (SSP) Programs and listed in the International Studbook (Hermes *et al.*, 2006a). The vagina consists of a thick muscular collapsed tube. The longitudinal wrinkling seen in the caudal vagina progresses to long and thin transverse folds that are contiguous with the cervical folds and frequently cover the external uterine ostium (Schaffer *et al.*, 2001a). Reported vaginal length of the Sumatran rhinoceros is around 18,0 cm with 5,0 to 8,0 cm in virtual diameter (Zahari, 1995). The cervix of a rhinoceros is located over the pelvic brim and consists of a complex system of thick fibrous folds. The folds are projected caudally and tend to be annular in the beginning and in the end of the cervix. In the middle of the cervix 3 to 5 folds interdigitate tightly, creating 90º to 180º turns and blind pockets. Both the external and internal uterine ostia are undefined due to vaginal and cervical folds surrounding the openings. The cervix of a rhinoceros is only patent during estrus (Schaffer & Beehler, 1990; Schaffer *et al.*, 2001a; Hermes & Hildebrandt, 2012). In the Sumatran rhinoceros the cervix is reported to measure between 6,0 and 8,0 cm in length and 2,0 to 5,0 cm in diameter (Zahari, 1995; Schaffer, Zahari, Suri, Jainudeen & Jeyendran, 1994). The rhinoceros uterus consists of a short body and long uterine horns, which are loosely fixed to the dorsal abdominal wall by the mesometrium (Schaffer *et al.*, 2001a). The tubular horns first run alongside, united by a muscular layer and a prominent dorsal intercornual ligament, giving the impression of a longer body. After the external bifurcation the horns run craniolaterally (Schaffer *et al.*, 1994; Schaffer *et al.*, 2001a). Both the body and the uterine horns are longitudinally plicated, and the endometrium holds abundant coiled tubular glands uniformly distributed (Schaffer *et al.*, 2001a). In the Sumatran rhinoceros reported lengths of the uterine body range from 2,5 to 4,5 cm with a diameter of 2,0 to 3,0 cm. In addition, a 0,5 cm thickness of the myometrium has been reported for the uterine body (Zahari, 1995). The uterine horns of the Sumatran rhinoceros measure from a reported 15,5 cm to the more common 30,0 to 34,0 cm with a diameter of 1,5 to 2,5 cm. A myometrial thickness of 0,1 to 0,4 cm has been reported for the uterine horns of Sumatran rhinoceroses (Zahari, 1995; Schaffer *et al.*, 1994; Furley, 1993). After the blunt cranial end of the uterine horns, the long and narrow uterine tubes or salpinges run in a convoluted way supported by the mesosalpinx. In the Sumatran rhinoceros these structures measure over 20,0 cm when uncoiled and the fimbriated infundibulum covers the ovaries (Zahari, 1995). The ovaries are suspended by the mesovarium and the suspensory ligament of the ovary, which houses the ovarian vessels. The mesovarium and the mesosalpinx form a pocket called ovarian bursa where the ovary lays, right after the end of the uterine horns and in close proximity to the colon (Schaffer *et al.*, 2001a; Zahari, 1995). Rhinoceros ovaries vary from round to flat and elongated, and their size and functional structures differ greatly among species. In the Sumatran rhinoceros the ovaries are usually ovoid and range from 4,0 to 8,0 cm in length and from 1,5 to 5,0 cm in width (Schaffer *et al.*, 1994; Zahari, 1995; Zahari, Jainudeen & Samsuddin, 1993; Roth *et al.*, 2001). Follicles can reach a diameter of 2,8 to 3,2 cm in the Sumatran rhinoceros (Schaffer *et al.*, 1994; Zahari, 1995) although preovulatory dimensions are considered to be 2,0 to 2,5 cm (Roth *et al.*, 2001). Follicles are usually spherical but irregular shapes can also be observed as a consequence of compression by adjacent functional structures (Zahari, 1995). Unlike the horse (the closest related domestic species), surface follicles and *corpora lutea* can be prominent in the rhinoceros, and follicles rupture on the surface of the ovary (Schaffer *et al.*, 2001a; Zahari, 1995). *Corpora lutea* in the Sumatran rhinoceros can reach a reported 3,5 cm in diameter (Zahari, 1995), and structures resembling *corpora albicantia* have been observed in White and Black rhinoceroses (Schaffer *et al.*, 2001a).

2.1. Clinical examination of the reproductive organs

In all but the smaller Sumatran rhinoceroses, dimensions and weight of the female reproductive tract in association with thickness of the rectal wall make it close to impossible to perform transrectal manipulation (Schaffer *et al.*, 1994; Schaffer *et al.*, 2001a; Hildebrandt, personal communication). Consequently, clinical evaluation of the reproductive tract in female rhinoceroses is commonly achieved by the use of transrectal ultrasonography, which allows for an efficient assessment of form, dimension, location and relative relationships of the reproductive organs, and also for an accurate determination of reproductive status and function, including ovarian cycle activity and pregnancy (Hermes & Hildebrandt, 2012; Schaffer *et al.*, 1994; Schaffer *et al.*, 2001a; Radcliffe, Czekala & Osofsky, 1997; Stoops, Pairan & Roth, 2004; Roth *et al.*, 2001; Adams, Plotka, Asa & Ginther, 1991). Portable ultrasound systems equipped with 2 to 8 MHz probes are sufficient for diagnostic imaging in these animals (Hermes & Hildebrandt, 2012). While in the Sumatran rhinoceros reported vulva-ovary distances are only 50 to 55 cm, in the larger species it often exceeds the arm reach of the examiner. In these cases, extensions of the ultrasound probes are necessary to assess the ovaries and anterior segments of the uterus (Schaffer *et al.*, 1994; Hermes & Hildebrandt, 2012; Schaffer & Beehler, 1990; Schaffer *et al.*, 2001a). Unlike horses, the risk of rectal perforation in rhinoceroses due to the use of extensions is believed to be minimal (Adams *et al.,* 1991; Schaffer *et al.*, 1994). Ultrasound examination of the rhinoceros reproductive tract can be performed using operant conditioning, standing sedation or full anesthesia (Hermes & Hildebrandt, 2012; Miller & Buss, 2015). Sumatran rhinoceroses seem to be particularly tolerant to human contact and invasive procedures, since several reports indicate that wild caught individuals are easily trained to stand in restraining chutes and accept transrectal examinations and even semen collections (Schaffer *et al.*, 1994; Zahari, 1995; Roth *et al.*, 2001; Agil, Supriatna, Purwantara & Candra, 2008). Another theoretically possible approach to the evaluation of the reproductive tract of female rhinoceroses would be the paralumbar access, either through laparotomy or laparoscopy. This option was studied in deceased White rhinoceroses by Schaffer *et al.* (2001a), who concluded that it is possible to reach and manipulate the horns of the uterus but not the ovaries due to their cranial position, the caudal extent of the ribs, the non-pliable nature of the skin, and the position and volume of the intestines. Furthermore, abdominal surgeries in rhinoceroses have so far been unsuccessful with inevitable dehiscence of the sutures due to thickness (reaching 5,0 cm) and inelasticity of the skin and high abdominal pressure (Miller & Buss, 2015; Valverde *et al.*, 2010). Laparoscopy has been used successfully (although with great difficulty) to access the uterus of a White rhinoceros and collect biopsies. Also on this attempt, the ovaries were impossible to reach or visualize (Radcliffe, Hendrickson, Richardson, Zuba & Radcliffe, 2000).

3. Reproductive physiology of the female rhinoceros

3.1. Estrous cycle

Rhinoceroses are believed to be polyestrous non-seasonal breeders, both in the wild and in captive conditions, although reproductive success can be impaired by high temperatures and food availability (Garnier, Holt & Watson, 2002; Kretzschmar, Ganslosser & Dehnhard, 2004; Patton *et al.*, 1999; Brown, Bellem, Fouraker, Wildt & Roth, 2001). Estrous cycle length has been characterized in four of the five rhinoceros species through the evaluation of progesterone and estrogen metabolites in feces, urine and serum samples. Although reports span over the last three decades, uncertainty and controversy still surround the normal cycle length for all species. For Black, White and Indian rhinoceroses, authors have consistently reported lengths of approximately 25 to 35 days and 55 to 85 days, but different interpretations have dictated the apparent inter-species differences assumed today. While in the Black rhinoceros short cycles (around 26 days) are believed to be normal and long cycles (twice the length) associated with pathological alterations (Hindle, Möstl & Hodges, 1992; Schwarzenberger, Francke & Göltenboth, 1993a; Berkeley, Kirkpatrick, Schaffer, Bryant & Threlfall, 1997; Radcliffe, Eyres, Patton, Czekala & Emslie, 2001; Brown *et al.*, 2001; Garnier *et al.*, 2002); in the White rhinoceros both short and long cycles are believed to be normal (30 to 35 days and 65 to 70 days - Hindle *et al.*, 1992; Schwarzenberger, Tomasova, Walzer & Möstl, 1993b; Schwarzenberger *et al.*, 1998; Radcliffe *et al.*, 1997; Patton *et al.*, 1999; Brown *et al.*, 2001); and in the Indian rhinoceros the largest variation of all is accepted as normal (36 to 86 days) with most cycles being reported around average values (43 to 48 days - Kassam & Lasley, 1981; Kasman, Ramsay & Lasley, 1986; Schwarzenberger *et al.*, 2000; Gomez, Jewell, Walker & Brown, 2004; Stoops *et al.*, 2004). In both Black and White rhinoceros authors have established a relationship between long cycles and either prolonged luteal phase or prolonged follicular phase. In several of the studied animals, prolonged luteal phase was found to be caused by early embryonic loss (Brown *et al.,* 2001; Garnier *et al.,* 2002; Radcliffe *et al.*, 1997; Patton *et al.*, 1999) sometimes due to uterine pathology such as endometritis and pyometra. Furthermore, a careful analysis revealed that for the majority of female White rhinoceroses reported to exhibit long cycles in a normal way, mating was either recorded or possible. Prolonged follicular phase has also been described as the reason behind long cycles, and is nowadays noted as the first sign of reproductive ageing and reduced fertility (Radcliffe *et al.*, 1998; Hermes, Göritz, Streich & Hildebrandt, 2007; Hermes & Hildebrandt, 2012). In regard to the Sumatran rhinoceros, three reports have placed the normal estrous cycle length between 21 and 25 days (Schaffer *et al.*, 1994; Heistermann, Agil, Büthe & Hodges, 1998; Roth *et al.*, 2001), with longer cycles being considered abnormal by the authors.

3.1.1. Behavioural estrus

Estrus duration can be assessed by changed behaviour and/or receptivity to a male, although altered behaviour often lasts longer than true sexual receptivity. Behavioural estrus is reported to last up to 3 or 5 days especially in the Black and Indian rhinoceroses (Garnier *et al.*, 2002; Kassam & Lasley, 1981; Gomez *et al.*, 2004), but mating encounters are often limited to one day of the cycle (Radcliffe *et al.*, 1997; Kassam & Lasley, 1981). The Sumatran rhinoceros appears to follow this trend, with receptivity to the male being typically limited to one day (Zahari, 1995; Roth *et al.*, 2001), although mating events spanning over 3 days have been reported in captivity (Mohamad & Romo, 2003). Changes of the external genitalia associated with estrus are small and subtle in all species of rhinoceroses (Hermes & Hildebrandt, 2012).

3.2. Ovarian dynamics

3.2.1. Functional structures

The functional structures of the ovary differ greatly among rhinoceros species. While the Graafian or preovulatory follicle is reported to measure approximately 3,0 cm in the White and 5,0 cm in the Black rhinoceroses, the Indian rhinoceros presents the largest follicle reported in a mammal with 12,0 cm in diameter (Radcliffe *et al.*, 1997; Hildebrandt *et al.*, 2007; Radcliffe *et al.*, 2001; Stoops *et al.*, 2004). In the Sumatran rhinoceros follicles can reach a diameter of 2,8 to 3,2 cm (Schaffer *et al.*, 1994; Zahari, 1995) although preovulatory dimensions are considered to be 2,0 to 2,5 cm (Roth *et al.*, 2001). Usually only one preovulatory follicle develops in each estrous cycle, alternating between ovaries in an apparently random way (Radcliffe *et al.*, 1997; Stoops *et al.*, 2004; Roth *et al.*, 2001). On at least one occasion however, two dominant follicles were observed in a Sumatran rhinoceros, one in each ovary, and both proceeded to ovulate (Roth *et al.*, 2001; Roth, Bateman, Kroll, Steinetz & Reinhart, 2004). The growth rate of dominant follicles has been described only for Black and White rhinoceroses as approximately 0,3 cm/day and 0,2 cm/day respectively. Also in these species an observable change in follicular shape from spherical to pear-shape has been noted up to 48h before ovulation (Radcliffe *et al.*, 1997; Radcliffe *et al.*, 2001; Hildebrandt *et al.*, 2007). This alteration was not observed in the Indian rhinoceros (Stoops *et al.*, 2004), and is not mentioned in literature regarding the Sumatran rhinoceros.

Ovulation has been detected in all rhinoceros species through the use of transrectal ultrasonography. Several authors identify ovulation as the collapse of a preovulatory follicle followed by the formation of a luteal gland, placing it between 24h and 48 to 72h after the onset of behavioural estrus (Radcliffe *et al.*, 1997; Radcliffe *et al.*, 2001; Roth *et al.*, 2001; Roth *et al.*, 2004; Stoops *et al.*, 2004).

Two types of luteal structures have been identified following the collapse of a dominant follicle. The *corpus hemorrhagicum* (CH) forms up to one day after ovulation, when the collapsed follicle fills with blood that later gets organized. There is a resulting central clot that remains discernible throughout luteal life (Radcliffe *et al.*, 1997; Stoops *et al.*, 2004). The other type, the proper *corpus luteum* (CL), presumably lacks the hemorrhagic event and therefore has a more uniform appearance with no central clot (Radcliffe *et al.*, 1997). It is thought to be discernible between 2 and 4 days after ovulation (Roth *et al.*, 2001). Interestingly, while Radcliffe *et al.* (1997) observed the two types of structures being formed at an approximate even ratio in the White rhinoceros, Stoops *et al.* (2004) claims that ovulation was consistently followed by the development of CH in the Indian rhinoceros. In the Sumatran rhinoceros, Roth *et al.* (2001) reported the formation of a CH by the ovulating follicle was never observed.

3.2.2. Hormone production and estrous cycle phases

In general, a follicular phase and a luteal phase are described across rhinoceros species by combining hormonal profiles with ultrasonographic findings. In all reports on White, Black and Sumatran rhinoceroses, progesterone levels go down before the onset of behavioural estrus and suffer a rapid increase few days after mating (Schwarzenberger *et al.*, 1993a; Brown *et al.*, 2001; Hindle *et al.*, 1992; Radcliffe *et al.*, 2001). In regard to the Sumatran rhinoceros, Roth *et al.* observed in 2001 serum progesterone levels rising between 3 and 5 days after estrus/mating and decreasing by day 19 to 21 in cycles of 21 days. This information can be translated to a luteal phase of 14 to 18 days, and the knowledge that progesterone drops between 1 and 2 days before the onset of estrus. In contrast with all other species, the Indian rhinoceros suffers an early decrease in progesterone values, followed by a diestrus phase with basal steroid levels (described only in this species) and a slight increase during the days preceding ovulation (Kasman *et al.*, 1986; Schwarzenberger *et al.*, 2000). There are fewer accounts on estrogen levels, but urinary estradiol is reported to peak on the day of behavioural estrus in all rhinoceros species (or the day before in some occasions) and then suffer a precipitous decline (Hindle *et al.*, 1992; Kassam & Lasley, 1981; Stoops *et al.*, 2004; Heisterman *et al.*, 1998).

3.3. Induced ovulation in the Sumatran rhinoceros

In disagreement with other rhinoceros species and all of the Perissodactyla (equids and tapirs), the Sumatran rhinoceros is believed to be an induced ovulator (Roth *et al.*, 2001; Roth, 2006). After almost two years studying one cycling female Sumatran rhinoceros, Roth and fellow authors reached this conclusion in 2001 based on three main arguments:

1) Ovulation was confirmed after all mating encounters, and failed to happen when estrus was predicted but the animals were not paired. Ovulation occurred within 46h after mating.

2) When mating did not take place, hemorrhagic anovulatory follicles were formed: follicles grew past preovulatory size and echogenic specks could be seen in the follicular fluid, followed by fibrinous quivering bands. According to the author, *corpora lutea* were not observed during the periods when mating was not allowed.

3) The concentration of luteinizing hormone (LH) in the serum was baseline before mating, increased approximately 30-fold within 1 to 2h of intromission and returned to baseline within 22h. When mating was not allowed, serum LH concentration was monitored for 6 days around the time of expected estrus, but did not increase above baseline.

Roth's theory gained significant strength when the first ever Sumatran rhinoceros was conceived and born in captivity in that same year, due to the methods described by the author. Regardless of merit, further notes should be taken on the paper at hand to allow for later analysis of Roth's theory. There were 11 mating encounters that resulted in confirmed ovulation, versus 3 times that estrus was expected but mating did not take place. The reasons why the animals were not paired on those days are not reported, except for day 348 when severe weather made it impossible. Furthermore, on 2 of the mating encounters successful intromission was not achieved. The author states that therefore, both mechanical stimulation (from mounting and partial intromission) and exposure to seminal fluid are possible mechanisms for inducing ovulation in this species. On another topic, it is stated that the female frequently developed anovulatory follicles throughout the year and that they were formed "most frequently" when the animals were not mated. When mating did not take place, serum progesterone and fecal progestin concentrations reached values similar to those of a true luteal phase, although with more erratic profiles. Consequently, the author classified the observed hemorrhagic anovulatory follicles as luteinized follicles. Regarding serum LH concentration, measurements were performed before and after mating on 2 occasions (days 327 and 535). On those days, serum LH concentration was baseline 1 to 2 h before copulation, and increased approximately 7-fold 4 h after copulation on day 327, and over 38-fold 1 h after copulation on day 535. On the other hand, one time that ovulation was expected but pairing of the animals was impossible due to rigorous weather conditions (day 348), serum LH concentration was monitored for 6 days (between day 347 and day 352) but did not increase above baseline.

4. Reproductive anomalies and pathology of the female rhinoceros

Anestrus and reproductive pathology are common findings in all studied species of rhinoceroses. Four common scenarios have been consistently reported through time: young females which fail to ovulate, middle-aged females with inactive ovaries, females with cervical and uterine leiomyomas, and females with endometrial cystic hyperplasia. A relationship between these findings was established in 2004 by Hermes, Hildebrandt and Göritz, who described the phenomenon of "asymmetric reproductive ageing" as a consequence of long non-reproductive periods and continuous ovarian cyclic activity. This degenerative syndrome comprises the development of reproductive pathology, depletion of follicular stock and premature senescence; and poses a serious threat to reproductive health and to the sustainability of captive breeding programs as supported by follow-up studies (Hermes *et al.*, 2006a; Hermes *et al.*, 2007; Hermes, Göritz, Saragusty, Stoops & Hildebrandt, 2014).

4.1. Anestrus

Anestrus is a common finding in captive rhinoceroses, as defined by lack of ovulation, acyclicity, and/or irregular or absent luteal activity. In the White rhinoceros specifically, several studies have revealed that approximately 50% of captive females are affected by it (Brown *et al.*, 2001; Patton *et al.*, 1999; Schwarzenberger *et al.*, 1998; Hermes *et al.*, 2001b; Hermes *et al.*, 2006a). Across the three most studied species, two different scenarios are commonly diagnosed: young females with dynamic follicular development but no ovulation, and middle-aged females with small inactive ovaries. Other reported reproductive anomalies associated with anestrus are persistent luteal structures, follicular cysts and para-ovarian tumours (Hermes *et al.*, 2001b; Hermes *et al.*, 2004).

<u>*4.1.1. Young females which fail to ovulate*</u>

Young rhinoceros females in anestrus exhibit regular follicular waves but produce dominant follicles that fail to ovulate, developing instead hemorrhagic anovulatory follicles or HAFs. Current scientific data, in addition to occasional reports of young females resuming normal cycle activity after introduction of new males or translocation to different facilities, indicate that inadequate animal husbandry and/or management are the primary causes for anestrus in young rhinoceros females (Hermes & Hildebrandt, 2012; Hermes *et al.*, 2007).

<u>*Hemorrhagic anovulatory follicles*</u>

Hemorrhagic anovulatory follicles or HAFs have been identified by several authors in the studied rhinoceros species and are regarded as non-physiological in all but the Sumatran rhinoceros. Reported diagnoses were based on one or more of the following features: persistence or growth of maximum follicle diameter following behavioural estrus; absence of follicular collapse; increased echogenicity of the follicular fluid; presence of fibrinous bands in the antrum; swirling of the follicular fluid and quivering of the echogenic bands upon ballottement; and/or failure in the development of a *corpus luteum* (Hermes *et al.*, 2006a; Radcliffe *et al.*, 1997; Radcliffe *et al.*, 2001; Stoops *et al.*, 2004; Roth *et al.*, 2004; Roth *et al.*, 2001). Although originally described in females without luteal activity (Radcliffe *et al.*, 1997; Hermes *et al.*, 2001b), hemorrhagic anovulatory follicles are often associated with progesterone production, which implies at least partial luteinization of the follicles. Most authors admit that anovulatory cycles have similar length and progesterone profiles to those of ovulatory cycles, although frequently more irregular or exhibiting lower peak concentrations (Roth *et al.*, 2001; Stoops *et al.*, 2004; Radcliffe *et al.*, 2001; Roth, 2006). Furthermore, some authors claim that HAFs are difficult to distinguish on ultrasonography from *corpora hemorrhagica* initially and *corpora lutea* once they have aged, basing the diagnosis on the increasing size of the anovulatory follicle and irregular production of progesterone (Radcliffe *et al.*, 1997) and even declaring the diagnosis unreliable without the use of serial ultrasonography (Roth, 2006). All authors have correlated these structures with equine HAFs, which are known to occur in the transitional phases when a mare is approaching or coming out of seasonal anestrus. Ginther, Gastal, Gastal and Beg reported in 2007 that equine HAFs originate from follicles with unaltered structure and viable cells, since ovulating and anovulating follicles differ in only two parameters. First, anovulating follicles present a higher percentage of follicle wall with colour Doppler signals on day -1, meaning that vascularization in the area of expected ovulation is greater in future HAFs thus leading to a larger hemorrhage upon rupture of the follicle. Second, a higher concentration of systemic estradiol was noted on day -3, and a relationship between elevated estradiol and increased vascularization was hypothesized.

4.1.2. Middle-aged females with inactive ovaries

Over several years, the high follicular output seen in female rhinoceroses which are not given the opportunity to breed, or which fail to ovulate like described in the previous point, leads to follicular depletion amongst other consequences. To illustrate, a healthy White rhinoceros female can produce up to 9 calves in captivity until the approximate age of 40. Considering a normal gestational period of 16 months plus a lactational anestrus of 12 months she would go through approximately 90 estrous cycles in her reproductive lifespan. If the female is not breeding however, those 90 cycles will have been displayed by the age of 15, the same age at which the first signs of pathological alterations are reported (Hermes *et al.*, 2004). Non-reproducing middle-aged females are therefore frequently affected by irregular follicular development and erratic luteal activity, which worsen as the follicular stock gets depleted, and ultimately cease completely. The ovaries become small and inactive, and premature reproductive senescence sets in 15 to 20 years ahead of the natural reproductive lifespan of a female rhinoceros. It is therefore understandable that changes in husbandry and various hormonal protocols have failed to re-establish ovarian activity in acyclic middle-aged females without a breeding history (Hermes *et al.*, 2004; Hermes *et al.*, 2006a).

4.2. Reproductive tract pathology

Pathological alterations are frequently found in the reproductive tract of female rhinoceroses including vaginal, cervical, uterine and ovarian tumours (leiomyomas, adenomas and adenocarcinomas); endometrial and ovarian cysts; endometrial cystic hyperplasia; and hydromucometra. The vast majority of pathological findings consists however in cervical and uterine leiomyomas and endometrial cystic hyperplasia. Infectious diseases of the reproductive tract are rarely described. Endometritis by *Pseudomonas* and hemolytic *Streptococcus* has been reported in Indian rhinoceroses with purulent vaginal discharge as the only clinical sign (Hermes & Hildebrandt, 2012).

4.2.1. Leiomyomas and endometrial cystic hyperplasia

Also a consequence of long periods without breeding is the development of reproductive pathology due to continuous ovarian cyclic activity and prolonged exposure of reproductive organs to sex steroid hormones (Hermes *et al.*, 2004). Leiomyomas of the cervix and uterus and endometrial cystic hyperplasia affect between 75% and 100% of captive White and Indian rhinoceroses over the age of 13 years, rendering half of them infertile even at an early age. Not only nulliparous females are affected by reproductive pathology, but also parous females which were either late breeders or had long intervals between calves (Hermes *et al.*, 2006a; Hermes *et al.*, 2014). Current scientific data indicates that pregnancy at an early age and continuous breeding prevent the development of reproductive pathology, and help maintain the fecundity of rhinoceros females throughout their natural reproductive lifespan (Hermes *et al.*, 2016).
In what concerns the Sumatran rhinoceros, going back in time it is possible to find several reports of reproductive pathology affecting captive and wild animals. In 2001 Schaffer, Agil and Bosi reported that of 17 wild-caught females examined since 1984 at least 50% suffered from uterine leiomyomas and cysts, starting at approximately 10 years of age and becoming prevalent in animals over 15. Also Ahmad *et al.* (2013) mentioned that in Malaysia 6 out of 9 captive females exhibited reproductive pathology.

Leiomyomas of the reproductive tract

The leiomyoma is the most frequently found reproductive tumour across all rhinoceros species, and has a higher prevalence among Indian rhinoceroses. The location of these benign, smooth muscle tumours within the reproductive tract is dependent on the species, with Indian rhinoceroses exhibiting them most frequently in the cervix and vagina while African species develop them essentially in the uterus (Hermes & Hildebrandt, 2012). Leiomyomas start as single small tumours of low echogenicity but soon increase in number and size, developing a detectable internal blood supply and hyperechogenic areas of necrosis. This process is largely asymptomatic until very advanced stages when copious bloody vaginal discharge appears and tumours may be seen protruding from the vagina. Previous discreet signs might have been pain reactions when mating, aggressive behaviour, conception failure, miscarriage, stillbirth, bloody discharge, anemia, and problems related to urination or defecation; as consequence of single large tumours, tumour necrosis, complete vaginal or cervical obstruction, and compression of ureters, urethra or rectum (Hermes *et al.*, 2016).

Endometrial cystic hyperplasia

Cystic hyperplasia of the endometrium occurs most frequently in African species. This pathological alteration also starts with single endometrial cysts, but soon evolves to multiple diffuse or confluent cysts. In later stages endometrial fibrosis develops and complications such as hydromucometra may occur (Hermes *et al.*, 2006a; Hermes & Hildebrandt, 2012).

5. Assisted reproduction of the female rhinoceros

5.1. Hormonal treatments

Due to the common finding of anestrus in captive female rhinoceroses, several protocols for hormonal induction of estrous cyclicity and ovulation have been attempted in the last decades. Various combinations of synthetic progestins (namely altrenogest) with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), follicle stimulating hormone (FSH), equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG) and gonadotropin releasing hormone (GnRH) have failed (Hermes & Hildebrandt, 2012). To this point only combinations of the synthetic progestin chlormadinone acetate (CMA) with hCG or GnRH agonists (deslorelin) have succeeded in inducing estrus and ovulation in female rhinoceroses (Schwarzenberger *et al.*, 1998; Hermes *et al.*, 2006a; Hermes *et al.*, 2012). Short-term GnRH agonist implants have also been successful in inducing ovulation of naturally developed preovulatory follicles (Hildebrandt *et al.*, 2007; Hermes *et al.*, 2007), and superstimulation of anestrous females (Hermes *et al.*, 2009b). Long-acting GnRH agonist implants in their turn, have been shown to reinitiate ovarian activity in anestrous females after a suppression of approximately 6 months, with some long term improvements (Hermes *et al.*, 2003; Hermes *et al.*, 2006a). Information on the aforementioned protocols is summarized on table 1.

Table 1: Summary of hormonal treatments employed in rhinoceros females and reported in the literature. Aim of each protocol is mentioned in the dark line above. Hormonal agents: chlormadinone acetate (CMA), human chorionic gonadotropin (hCG), gonadotropin releasing hormone agonist deslorelin (GnRH). Abbreviations: preovulatory follicle (preov.).

Agents	Protocol	Follicles	Estrus/Ovulation	References
Induction of behavioral estrus and ovulation				
CMA	30 mg/ 36h x 45 days	preov. 80%	30%	Schwarzenberger *et al.*, 1998
hCG	10 000 IU			Hermes *et al.*, 2006a
CMA	30 mg/ 24h x 45 days	-	67%	Hermes *et al.*, 2012
-	9,5 days of pause			
hCG	10 000 IU			
CMA	30 mg/ 24h x 45 days	-	93%	
-	9,5 days of pause			
GnRH	4,2 mg deslorelin (48h)			
Induction of ovulation of a preovulatory follicle				
GnRH	4,2 mg deslorelin (48h)	-	80% in 48h	Hildebrandt *et al.*, 2007
				Hermes *et al.*, 2007
Superstimulation of anestrous females				
GnRH	4,2 mg deslorelin (48h) /48h x 3	28 - 34 >0,5 cm	-	Hermes *et al.*, 2009b
Induction of estrous cyclicity in acyclic females				
GnRH	14,1 mg deslorelin (6-8 months)	up to 4 >1,5 cm	0%	Hermes *et al.*, 2003
				Hermes *et al.*, 2006a

In regard to the last mentioned protocol (long-acting GnRH agonist implants), during downregulation the authors observed a reduction in follicular cysts, endometrial cystic hyperplasia and even leiomyoma dimensions (Hermes *et al.*, 2003; Hermes *et al.*, 2006a). This revelation was later explored as a treatment option for reproductive pathology. In 2016 Hermes and colleagues published a study focusing on GnRF (gonadotropin releasing factor) vaccination as a way of downregulating reproductive function and consequently reducing the extension of reproductive pathology and associated clinical signs. Females of the Indian and White species bearing extensive reproductive tumours and clinical signs were vaccinated according to the protocol on table 2. Results showed a significant reduction in ovarian activity followed by cessation of bloody vaginal discharge, reduction of approximately 50% in calculated tumour volume for both species, and lack of development of new tumours for up to 16 months after vaccination (Hermes *et al.*, 2016).

Table 2: Summary of vaccination protocol against gonadotropin releasing factor (GnRF). Aim of the protocol is mentioned in the dark line above.

Agent	Protocol	Reference
Downregulation of reproductive function and reduction of reproductive pathology		
vaccination with synthetic analogue of GnRF	450 µg at 0, 4 and 16 weeks	Hermes *et al.*, 2016
	boosters every 6 to 8 months	

5.2. Artificial insemination

The first report of artificial insemination (AI) in rhinoceroses was provided by Hermes and colleagues in 2000(a). At that time the team had already understood the need for a special catheter in order to perform non-surgical AI, and patented the idea. The initial attempts were performed with the guidance of endoscopy and transrectal ultrasonography, and allowed for cervical and/or uterine deposition of sperm. Hildebrandt *et al.* reported in 2002 that none of the initial attempts was successful due to difficulties in timing the procedure in relation to ovulation. After the original technique was improved and a protocol to reliably induce ovulation was established, the first birth of a rhinoceros conceived by AI was achieved in 2007, followed by the first conception and birth using frozen sperm in 2009. The improved method was described as follows. After a close study of reproductive health and activity, artificial insemination is performed using a catheter specially designed to overcome the two main anatomical obstacles of the rhinoceros reproductive tract: the hymeneal membrane of nulliparous females and the convoluted cervix with its numerous tight folds. The patented catheter consists of an external flexible carbon sheath measuring 115 cm with a handle in the proximal end and a smooth, angled stainless-steel tip in the distal end. On the inside the catheter holds a sterile 90 cm Luer-lock cannula. The sterile catheter is guided into the vagina by digital palpation, and the cervix is transrectally fixed while the catheter is manoeuvred through the cervical folds. Transrectal ultrasonography is used to guide the catheter tip into the uterine horn on the side of expected ovulation. The sterile cannula is then employed to deposit either fresh semen (extended 1:1 and warmed to 37ºC) or high quality cryopreserved sperm after thawing (Hildebrandt *et al.*, 2007; Hermes *et al.*, 2009a). By 2012 a total of five rhinoceroses were born after conception by AI (Hermes & Hildebrandt, 2012).

5.3. Oocyte collection and in vitro techniques

In infertile female rhinoceroses suffering from extensive reproductive pathology, oocyte collection and *in vitro* techniques represent the only option for contribution to the genetics of a population. Originally the collection of oocytes was only possible from deceased rhinoceroses, as *in vivo* techniques such as laparoscopic and transvaginal endoscopic collection proved to be impractical mostly due to the length of the reproductive tract, inability to transrectally fixate the ovaries, and difficulty in insufflating the abdomen (Hermes *et al.*, 2007). A new *in vivo* technique which led to successful *in vitro* fertilization (IVF) and the first rhinoceros embryo ever produced *in vitro,* was reported in 2009 by Hermes and colleagues (b). The technique termed transrectal “ovum pick-up” (OPU) consists in the aspiration of ovarian follicles through transrectal access using ultrasound guidance, and required the development of a special apparatus to hold both the ultrasound transducer and a specialized needle. As described by Hermes *et al.* 2009b, the procedure starts with the thorough cleaning of the rectum using manual removal of feces, multiple enemas, and a povidone-iodine solution to soak and flush the rectal ampulla. Sterile towels are placed cranially in the rectum, and the perineal area is scrubbed. A custom-made single-lumen needle with 100 cm and 14 gauge is mounted onto the device that also holds a micro-convex ultrasound transducer, and the set is introduced in the rectum using sterile lubricant. The ovary is then visualized with ultrasonography and placed next to the rectum, and the needle forced through the rectal wall into the follicle. Follicular fluid is aspirated and the follicle repeatedly flushed under ultrasound visualization. *Cumulus*-oocyte complexes (COCs) are then recovered with specialized filters and identified using stereoscopic microscopy, to which follow IVM (*in vitro* maturation), IVF and/or ICSI (intracytoplasmic sperm injection) techniques. Post-surgical wound management includes antibacterial foam sticks placed in the rectum and broad-spectrum systemic antibiotics. No post-surgical complications such as intra-abdominal adhesions or visible ovarian scar tissue were noted by the authors, even after repeated collection in the same animal (Hermes *et al.*, 2009b).

In what concerns the Sumatran rhinoceros, the first report of *in vitro* techniques being applied to the species came from Stoops, Bateman, Campbell and Roth in 2011. The team performed *post-mortem* collection of several oocytes from a multiparous female, although IVM was only successful in a small percentage of oocytes and IVF failed.

5.4. Cellular technologies

Among several cellular technologies applicable to rhinoceros conservation and described by Saragusty *et al.* in 2016, one stands out due to extensive efforts and preliminary positive results: artificial production of gametes by directed differentiation of pluripotent stem cells (PSCs). In 2006 Takahashi and Yamanaka described a transformative technology which enabled the cellular reprogramming of somatic cells such as skin fibroblasts into induced PSCs (iPSCs). These iPSCs are believed to have the same developmental potential as embryonic stem cells, and have been shown to be capable of generating all tissue types in mice, including functional gametes that could be used in assisted reproduction techniques (Saragusty *et al.*, 2016). Remarkably, iPSCs have been generated from fibroblast cultures of Northern White rhinoceros using retroviruses to deliver the reprogramming factors, which may lead to a series of problems (namely tumours) in later stages of the process. Further techniques of delivery developed for humans could be applied to rhinoceros cells in a near future (Saragusty *et al.*, 2016).

One other technology which has been studied for a long time is the possibility of sorting sperm by the sexual chromosome held in each cell. Sorted sperm would then be used with assisted reproduction techniques in order to create offspring that is sexually biased towards the females, and thus produce a larger number of individuals in a short time frame. Sperm sorting has been performed successfully in Black, White and Indian rhinoceroses, but so far conception has not been achieved (Saragusty *et al.*, 2016; Hermes & Hildebrandt, 2012).

6. Reproductive anatomy of the male rhinoceros

The first report on a double-horned rhinoceros in Asia, later known as the Sumatran rhinoceros, was written by Bell in 1793 and included a description of the male reproductive system. In 1880 a report focusing solely on the "male generative organs" was published by Forbes after the death of the first male Sumatran rhinoceros to reach Europe alive. As in other rhinoceros species, the external and internal genitalia consist of prepuce, penis, testicles, epididymides and accessory sex glands namely bulbourethral glands, prostate and seminal glands (Hermes & Hildebrandt, 2012). The penis is of the musculocavernous type, and when relaxed it is fully covered by the preputial fold. During urination and territorial marking the penis exits the sheath pointing caudally between the hind legs. During erection the glans swings forward until the fully erect penis points cranially (Hermes & Hildebrandt, 2012; Schaffer & Beehler, 1990). The rhinoceros penis presents two structural particularities which vary between species. The first is a pair of cavernous projections known as *processus glandis*, located near the basis of the *glans penis*. In the Indian, Javan, Black and White rhinoceroses these processes can be generally described as rounded to triangular flaps, with a long base of attachment in the lateral to dorsolateral aspects of the glans, protruding up to 25,0 cm laterally when erect in the live Black rhinoceros (Cave, 1964; Hermes & Hildebrandt, 2012; Schaffer & Beehler, 1990; Schaffer *et al.*, 2001a). The Sumatran rhinoceros however bears completely distinctive *processus glandis* (fig. 51). Several accounts have been made describing two long projections "very like the nipples of a milk cow", only attached at their base and located para-medially in the dorsal aspect of the erect glans (Bell, 1793; Forbes, 1880; Zahari, 1995). Dimensions were reported by Forbes (1880) and Zahari (1995) agreeing on approximately 6,0 cm in length by 3,5 to 4,0 cm in width, and located 16,5 to 18,0 cm from the tip of the glans. Zahari (1995) also claimed that during erection the projections expanded to twice the normal size. Both Zahari (1995) and Schaffer *et al.* (2001a) claimed the size and cranial projection of the *processus glandis* of the Sumatran rhinoceros are likely to inhibit intromission after full erection has been achieved. The second characteristic feature of the rhinoceros penis is the cup-like distal extremity of the glans, which encloses the *fossa terminalis* and the *processus urethralis* with the external urethral orifice. Both levels present everted edges giving the *processus urethralis* a mushroom-like appearance (Cave, 1964; Hermes & Hildebrandt, 2012; Bell, 1793; Forbes, 1880).

The testicles of the rhinoceros are extra-abdominal and located within the same skinfold as the penis. Their position and long axis shifts from horizontal, closer to the inguinal rings and therefore non-palpable; to more vertical and caudal, often visible and palpable (Hermes & Hildebrandt, 2012). In what concerns the Sumatra rhinoceros, authors disagree on the existence of a scrotal sac. While Bell (1793) and Forbes (1880) state that the testicles hardly appear externally due to an inexistent scrotum, Schaffer *et al.* (1994) and Zahari (1995) claim that the testicles are more visible than in other species due to a "pendulous scrotum" caudal to the penile sheath. The testicles of rhinoceroses are enclosed in thick connective tissue capsules, and their parenchyma is divided into lobules by a moderate amount of interstitial connective tissue (Schaffer *et al.*, 2001a). Short-axis cross-sectional ultrasound images of the testicles are reported to be circular and homogeneous in echotexture with small hyperechoic central areas (0,2 to 0,4 cm) corresponding to the *mediastinum testis*. Reported testicular measurements for Sumatran rhinoceroses obtained via ultrasonography and posthumously are 9,8 to 16,5 cm in length and 5,0 to 6,3 cm in width (Schaffer *et al.*, 1994; Zahari, 1995). The epididymides of the different rhinoceros species are reported to have varying degrees of testicular attachment (Schaffer *et al.*, 2001a), with those of Sumatran rhinoceroses being associated on several accounts to fluid accumulations surrounding the head and body. Although very common, these bilateral swellings are believed to be of pathological nature, and are therefore analysed ahead (page 26). Regarding epididymal dimensions in the Sumatran rhinoceros, the head was reported to measure between 1,5 and 2,5 cm in length and width, while the tail was said to measure between 3,0 and 4,0 cm in width and up to 5,5 cm in length (Schaffer *et al.*, 1994; Zahari, 1995). The *vas deferens* was described by Forbes (1880) in the same species as measuring 74,0 cm in length and 0,3 cm in diameter.

The accessory sex glands of the rhinoceros are the bulbourethral glands, the prostate and the seminal glands. *Ampullae* of the *vasa deferentia* are reported to be absent in all rhinoceros species (Schaffer & Beehler, 1990; Hermes & Hildebrandt, 2012). All accessory sex glands are located within the pelvic canal, in close association to the urinary system. Straight-line distance between neck of the bladder and anal sphincter in the Sumatran rhinoceros was reported to be approximately 15,0 to 20,0 cm (Zahari, 1995; Agil *et al.*, 2008). The paired bulbourethral glands are the most caudal accessory sex glands, just cranial to the anus. They are located dorsolaterally to the pelvic urethra and vary in size and shape between species. While in the Indian and Black rhinoceroses the bulbourethral glands are round and smaller, in the White and Sumatran rhinoceroses they are more elongated and irregular in shape. Using ultrasonography they appear compact, and in the Sumatran rhinoceros large blood vessels were reported crossing the parenchyma. A thick muscular layer surrounds the bulbourethral glands in all species

(Hermes & Hildebrandt, 2012; Schaffer & Beehler, 1990; Schaffer *et al.*, 1994; Schaffer *et al.*, 2001a). Measurements for the Sumatran rhinoceros are neglected in the literature except for Forbes (1880), who described the bulbourethral glands as large oval structures measuring 9,0 cm in length by 5,0 cm in width. Their ducts were observed to open in front of the ejaculatory ducts (Forbes, 1880). The prostate appears cranially, with two triangular lobes united by an isthmus. The prostate surrounds the terminal portion of the urethra (prostatic urethra) and extends over the neck of the bladder. In the rhinoceros, the prostatic parenchyma is divided into lobules by thick connective tissue septa, with the most pronounced lobular surface pattern being observed in the Indian species. The prostatic lobules may reach a cystic state when filled with prostatic fluid (Hermes & Hildebrandt, 2012; Schaffer & Beehler, 1990; Schaffer *et al.*, 1994; Schaffer *et al.*, 2001a). Schaffer & Beehler (1990) claim the prostate is often difficult to distinguish using ultrasonography due to its irregularity and uneven border. Measurements of the prostate were not found in the literature. The paired seminal glands (also termed seminal vesicles or vesicular glands) are elongated glands which can be cigar-shaped or more flattened. The seminal glands extend cranially and dorsally from the neck of the bladder, and often are partially covered by the prostate. They have a gross lobulated appearance and histologically are considered sacculated or multicystic. These sacculations can be filled with fluid, turning the gland hypo- to anechoic with hyperechoic specks under ultrasonography; or more empty and collapsed, looking irregularly hypo- to hyperechoic (Hermes & Hildebrandt, 2012; Schaffer & Beehler, 1990; Schaffer *et al.*, 1994; Schaffer *et al.*, 2001a; Zahari, 1995). Zahari (1995) described the seminal glands of the Sumatran species as flattened and elongated measuring approximately 2,4 cm in length. Forbes (1880) in his turn reported them as measuring 19,0 cm in length and 2,5 cm in width.

Regarding the pelvic urethra, Forbes (1880) described the *verumontanum* or *colliculus seminalis* of the Sumatran rhinoceros as a short and rounded structure measuring 1,3 to 2,5 cm. Here the very short ejaculatory ducts (resulting from the union of the *vasa deferentia* with the ducts of the seminal glands) opened to the urethra in two small pores, and a larger pore seen close above in the middle represented the *vesicula prostatica* or prostatic utricle. Schaffer, Bryant, Agnew, Meehan and Beehler (1998) described the *colliculus seminalis* as a dorsal bulge somewhere in the frontier between the caudal prostatic urethra and the cranial pelvic urethra ("just caudal to the cranial constriction", see ahead page 25) in White, Black and Indian rhinoceroses. However, Schaffer *et al.* (2001a) mentioned the *colliculus seminalis* held the openings for the ducts of seminal and prostate glands, and the openings of the *vasa deferentia*, suggesting there were no evident ejaculatory ducts in the three rhinoceros species. Schaffer & Beehler (1990) also acknowledge the existence of the masculine uterus, nowadays known as prostatic utricle, in the African rhinoceros (but apparently not in the Indian species). A progressive dilation of the pelvic urethra caudal to the prostate was reported by Schaffer *et al.* (1998, 2001a) in White, Black and Indian rhinoceroses. This structure is described as a flattened bulbous expansion of the urethra measuring approximately 2,0 cm in height and 5,0 cm in width, in a length of approximately 5,0 cm in Indian rhinoceros and 8,0 cm in White rhinoceros. This expansion is limited by a cranial constriction at the prostatic urethra and bladder neck, and by a caudal constriction of the pelvic urethra at the level of the bulbourethral glands.

6.1. Clinical examination of the reproductive organs

Testicular palpation to assess functional status or the existence of pathological alterations is of limited use in the various rhinoceros species due to the location and changing position of the testicles, thickness of the skin, dense testicular capsules and varying level of epididymal attachment (Hermes & Hildebrandt, 2012). In spite of being located in the pelvic canal just ventral to the rectum, accessory sex glands are also not palpable in the rhinoceros, except for the isthmus of the prostate according to Schaffer *et al.* 2001a. Consequently, clinical examination of the reproductive tract of male rhinoceroses relies on transrectal and transcutaneous ultrasonography, which allow for an accurate assessment of form, dimension, location, relative relationships, reproductive status and pathological changes (Hermes & Hildebrandt, 2012; Schaffer & Beehler, 1990; Schaffer *et al.*, 1994; Zahari, 1995). While transrectal ultrasound examination in male rhinoceroses requires sedation or operant conditioning and the use of a chute, transcutaneous ultrasonography of the testicles can be achieved with little training through the bars of the enclosure (Hermes & Hildebrandt, 2012). Ultrasound-guided fine-needle biopsy of the testicles was also reported by Hermes *et al.* (2006b) as a method of the assessment for spermatogenic activity of males with aspermatic ejaculates. The report concluded that valuable information could be obtained using this technique in combination with ultrasonographic examination of the reproductive organs.

7. Reproductive physiology of the male rhinoceros

Rhinoceroses are believed to be non-seasonal breeders as mating events and androgen production take place throughout the year (Kretzschmar *et al.*, 2004; Brown *et al.*, 2001; Hermes *et al.*, 2005). The two most important aspects of male rhinoceros reproductive physiology are the ejaculatory process and the characteristics of the ejaculate (see ahead page 29). The ejaculatory process of the male rhinoceros was described by Schaffer *et al.* in 1998 using penile massage and electroejaculation coupled with transrectal ultrasonography. According to the author, rhinoceroses exhibit the stages of ejaculation common to most mammals, starting with seminal emission and finishing with ejaculation proper. In the first stage termed seminal emission, fluids and spermatozoa are delivered from the accessory sex glands and *vasa deferentia* to the pelvic urethra. The respective ducts open at the *colliculus seminalis* and can be seen on ultrasound as multiple thin hypoechogenic lines. The author reported that neither the ducts nor the accessory sex glands change visibly after stimulation and emission. A urethral constriction at the level of the bladder neck and prostatic urethra, and cranial to the *colliculus seminalis* (the internal urethral sphincter) closes before emission preventing the passage of semen to the bladder and the contamination of semen with urine. A second more caudal urethral constriction at the level of the bulbourethral glands (the external urethral sphincter) also closes before emission, avoiding the outflow of semen to the penile urethra. In between these constrictions the bulbous dilation of the pelvic urethra gets filled with the products of emission, expanding laterally and longitudinally. Once the pelvic urethra is filled with semen, the caudal urethral constriction relaxes while the cranial constriction remains closed, initiating the second stage termed ejaculation proper. Clonic contractions of the *urethralis* and *bulbospongiosus* muscles along with other pelvic musculature result in the expulsion of semen from the pelvic and penile urethra to the outside (Schaffer *et al.,* 1998). Regarding the expansion of the pelvic urethra, Schaffer *et al.* (1998) refers to this structure as an equivalent to the “pressure chamber” mentioned in the human and dog, as the accumulation of semen theoretically optimizes contraction of the *urethralis* muscle at the time of ejaculation proper, adding force to the propulsion of semen through the length of the penile urethra. In regard to the site of semen deposition in the female rhinoceros during natural breeding, Schaffer *et al.* (1998) also speculated the pressure created in the pelvic urethra was necessary for the propulsion of semen “through the complicated cervix of the female”. Hermes and Hildebrandt (2012) further supported the theory of cervical deposition with basis on the anatomical particularities of the rhinoceros penis. According to the authors, during the long intromission of 45 to 60 minutes the lateral projections of the glans (*processus glandis*) expand inside the vagina presumably causing the cervix to become more patent, while the long cup-like *processus urethralis* locks into de external ostium and cervical folds (Hermes & Hildebrandt, 2012). As a final note on male reproductive physiology, it has been shown by Hermes and colleagues (2005) that the volume of the accessory sex glands in adult rhinoceroses is positively correlated with semen quality, namely the percentage of motile and intact spermatozoa. This finding suggests that reproductive fitness requires sufficiently developed accessory sex glands besides adequately stimulated spermatogenesis (Hermes *et al.*, 2005).

8. Reproductive anomalies and pathology of the male rhinoceros

Pathological alterations of the reproductive tract and function of male rhinoceroses can be of traumatic, neoplastic or degenerative nature (Hermes & Hildebrandt, 2012). Trauma to the penis during mating or masturbation can occur resulting in superficial abrasions and edema. If not promptly treated or if trauma is severe, further edema can prevent the retraction of the penis. In the unprotected hanging penis blood circulation is compromised and ulceration and permanent damage can occur. Trauma to the erect penis has also been reported to result in penile fracture with subsequent inability to mate (Hermes & Hildebrandt, 2012). In regard to the testicles, trauma can occur during territorial fighting and rough courtship, and affect the production of semen temporarily or permanently after the development of hematomas, seromas, permanent stenosis of the spermatic cord, and even testicular necrosis and atrophy (Hermes & Hildebrandt, 2012). Testicular neoplasias have been diagnosed during ultrasonographic assessments, followed by testicular biopsy or *post-mortem* analysis. Seminoma has been identified in both White and Black rhinoceroses, and once was resolved by hemicastration with subsequent maintenance of breeding potential (Portas *et al.*, 2005; Portas, Hildebrandt, Bryant, Göritz & Hermes, 2010). The most commonly described affliction of the testicles in rhinoceroses is diffuse multifocal testicular fibrosis. Using ultrasonography, fibrotic hyperechogenic spots measuring between 0,1 and 0,3 cm can be observed in the testicular parenchyma of males over 15 years of age. The size and number of these hyperechogenic *foci* have been positively correlated to age and are regarded as signs of testicular ageing (Hermes *et al.*, 2001a; Hermes *et al.*, 2005; Stoops, Atkinson, Blumer, Campbell & Roth, 2010). In spite of the degenerative nature of these alterations, semen production and quality are reported not to be affected as afflicted older males have shown high values of sperm concentration and motility (Hermes *et al.*, 2001a; Hermes *et al.*, 2005; Stoops *et al.*, 2010). Regarding alterations in size and consistency, reports disagree on whether age and/or sexual inactivity result in testicular atrophy (Hermes *et al.*, 2001a; Hermes *et al.*, 2005). Epididymal cysts have been reported in White and Sumatran rhinoceroses as fluid filled structures measuring between 1,0 and 10,0 cm and causing consistently aspermatic or very low quality ejaculates (Hermes & Hildebrandt, 2012; Hermes *et al.*, 2006b). In the case of this Sumatran rhinoceros, further examinations revealed the fluid was free to surround the epididymis, constituting an hydrocele rather than an epididymal cyst (Hildebrandt, personal communication). As previously mentioned, the epididymides of Sumatran rhinoceroses have been associated on several accounts to fluid accumulations surrounding the head and body. These bilateral swellings are said to be externally visible and palpable, and once were reported to measure 8,0 by 4,0 cm. Using ultrasonography it was possible to observe the anechoic accumulations surrounding the hypoechoic epididymis, measuring 0,5 to 0,8 cm between *tunica vaginalis* and *tunica albuginea.* At least one male Sumatran rhinoceros to which these reports refer is noted to be unable to breed (Schaffer *et al.*, 1994; Zahari, 1995). Pathological alterations of male accessory sex glands are thought to be rare, with prostatic cysts in one White rhinoceros being the only documented disorder (Hermes & Hildebrandt, 2012).

9. Assisted reproduction of the male rhinoceros

9.1. Semen collection

The assessment of reproductive potential of a male rhinoceros usually consists in two parts: the clinical examination of the reproductive organs, and the collection and evaluation of semen samples (Hermes & Hildebrandt, 2012; Hildebrandt, Hermes, Jewgenow & Göritz, 2000). In regard to semen collection, it can be performed following natural breeding or using alternative methods to stimulate ejaculation such as penile massage, artificial vagina, transrectal massage of the pelvic urethra and accessory sex glands, electroejaculation and combinations of the above (Platz, Scager & Bush, 1979; Schaffer, Beehler, Jeyendran & Balke, 1990; Zahari, 1995; O'Brien & Roth, 2000; Hermes *et al*., 2001a; Hermes *et al*., 2005).

Post-coital semen collection was performed 5 times in a Sumatran rhinoceros by capturing the fluid draining from the vulva, 20 to 180 minutes after copulation (O'Brien & Roth, 2000). This method requires first and foremost the presence of normal mating behaviour, intromission and ejaculation, which are often absent in males that require semen assessment. Furthermore, high quality spermatozoa are expected to stay inside the female reproductive tract; hygiene of the sample is questionable; and the authors reported large amounts of leucocytes (twice over 30% of all cells) presumably from female origin, reducing the quality of the sample (O'Brien & Roth, 2000; Hermes & Hildebrandt, 2012).

Manual stimulation can be applied to the penis directly (penile massage) and/or to the accessory sex glands and pelvic urethra through the rectal wall (transrectal or rectal massage). These techniques can be performed in highly trained or sedated animals, and have obtained results in all studied rhinoceros species (Schaffer *et al*., 1990; Schaffer *et al*., 1998; Zahari, 1995; Hermes *et al*., 2001a). However, semen collection by manual massage only is time consuming and often yields very poor or aspermatic samples (Hermes & Hildebrandt, 2012; Agil *et al*., 2008). According to Schaffer *et al.* (1998), while natural arousal, operant conditioning and electroejaculation result in emission of seminal fluid into the pelvic urethra, penile massage does not. This means that in manual collections a stimulus that incites seminal emission might be necessary in order to obtain true ejaculation (Schaffer *et al.*, 1998). Reports on this technique also mention a filamentous appearance of the semen, which microscopic analysis exposed as densely aggregated spermatozoa with 10-30% motility (Hermes *et al*., 2001a; Zahari, 1995). These filaments are believed to be moulds of the *vasa deferentia* which are passively emitted without further contribution from the epididymides and accessory sex glands (Hermes *et al*., 2001a; Schaffer *et al*., 1998).

Artificial vaginas are difficult to employ due to the natural curvature of the rhinoceros penis and its lateral projections, and maintaining the erection after fitting the apparatus has been proven difficult (Young, 1967; Schaffer *et al*., 1990). Alone they are largely unsuccessful (Hermes *et al*., 2001a). In combination with penile and sometimes rectal massage, artificial vaginas have been reported to yield results, although most often small volumes and very poor sperm concentrations (Young, 1967; Zahari, 1995; Schaffer *et al*., 1990; Agil, Supriatna & Purwantara, 2004; Agil *et al*., 2008).

Nowadays, electroejaculation is considered the method of choice for semen collection in all rhinoceros species, since it provides the most reliable sampling for semen evaluation and sperm preservation. Electroejaculation requires general anesthesia and therefore can be applied in untrained, captive or wild rhinoceroses (Hermes & Hildebrandt, 2012; Hermes *et al*., 2005). Upon electrostimulation, electric current induces contraction of the *vasa deferentia* and accessory sex glands resulting in emission, followed by contraction of the pelvic urethra. According to Schaffer *et al*. (1998), clonic contractions of the muscles responsible for the expulsion of semen from the urethra are initiated by penile massage, but not by electroejaculation alone. Manual massage of the pelvic and penile urethra is therefore recommended between electric stimulations to produce ejaculation proper (Hermes & Hildebrandt, 2012; Hermes *et al*., 2005; Schaffer *et al*., 1998). The first report of electroejaculation in a rhinoceros was provided by Platz *et al*. in 1979, who used rectal and penile electric stimulation. Schaffer *et al*. (1990) was the first to use an adapted electroejaculation rectal probe in combination with penile massage, obtaining less than 2,0 ml of semen but with high sperm concentration. Since then the method has been improved and semen samples up to 200 ml have been collected with high concentrations such as $80x10^6$ to $200x10^6$ spz/ml and up to 90% motility (Hermes & Hildebrandt, 2012; Hermes *et al*., 2009a; Hermes *et al*., 2005; Hermes *et al*., 2001a; Roth *et al*., 2005; Schaffer *et al*., 1998; Reid *et al*., 2009). The improved method as described by Hermes *et al.* (2005) uses a combination of electroejaculation with manual massaging of the accessory sex glands, pelvic urethra and penile urethra. The electrical probe should be adapted to cover the pelvic urethra and allow for the spreading of the rectal mucosa and maximum electric coupling. A total of 3 to 4 sets of 3 to 4 electrical stimuli is applied with increasing voltage (5-20 V) and amperage (200-900 mA). Each set of stimulations is followed by manual massage of both pelvic and penile urethra. Collection bags placed around the cleansed penis guide the semen into 50 ml aseptic tubes enclosed in dark foam insulators (Hermes *et al*., 2005). Previous ultrasonographic evaluation is helpful to determine the exact position of the accessory sex glands, allowing for the correct placement of the rectal probe and avoiding the stimulation of the bladder neck and subsequent contamination of the ejaculate (Hildebrandt *et al*., 2000; Hermes *et al.*, 2005). Urine contamination, identifiable due to changes in odour and colour, increased volume and decreased motility, is reported by Hermes *et al*. (2001a, 2005) as a possible side effect occurring in intermediate phases due to misplacement of the probe, or at the end of the procedure due to over-stimulation. As a last option, spermatozoa can be collected directly from the epididymides, usually after castration or unexpected death of the animals. Epididymal collection should be performed as soon as possible to avoid tissue and temperature changes that affect sperm quality. Transportation of testicles and epididymides in 4ºC cooled saline solution is an alternative to on-site collection. *Post-mortem* sperm extraction and cryopreservation have resulted in pregnancy after artificial insemination performed by Hermes and colleagues (Hermes & Hildebrandt, 2012; O'Brien & Roth, 2000; Saragusty, 2012).

9.2. Semen evaluation

The evaluation of semen samples includes the assessment of total volume, colour and odour, and sometimes pH and osmolarity; followed by the assessment of sperm concentration, motility, morphology and viability. Values obtained vary greatly between species and individuals, history of mating or masturbation, territorial behaviour and social structure, collection method, stimulation protocol, operator and others, and must therefore be judged carefully (Hermes & Hildebrandt, 2012; Hermes *et al*., 2005; Roth *et al*., 2005; O'Brien & Roth, 2000). Sperm motility, viability and morphology seem to be the most relevant indicators of semen quality; whereas total volume, pH and osmolarity don't seem to hold a significant correlation to quality. Sperm motility is therefore directly used to judge the fertilizing potential of a male, with over 75% being considered of excellent potential, 75% to 50% as intermediate, and under 50% perceived as poor. However, samples with 60% motility have been proven sufficient for artificial insemination resulting in conception (Hermes & Hildebrandt, 2012; Hermes *et al*., 2005). Table 3 reports all available information on Sumatran rhinoceros semen.

Table 3: Summary of published information regarding semen characteristics of Sumatran rhinoceroses, as reported by Zahari, 1995; Agil *et al.*, 2004; Agil *et al.*, 2008; O'Brien & Roth, 2000; Kretzschmar *et al.*, 2007; Hermes & Hildebrandt, 2012. Collection methods are penile massage (PM), transrectal massage of the accessory sex glands and pelvic urethra (RM), artificial vagina (AV), electroejaculation (EJ), post-coital collection (PC) and combinations of the above. Number of reports of each method or combination is mentioned under "n".

n	Collection method	Volume (ml)	Concentration ($x10^6$/ml)	Motility (%)	Normal morphology (%)
1	PM	1,5	0	-	-
1	PM + RM	0,25	1,9	40	30
1	AV + PM	1,5 - 2,1	0,14 - 0,33	very weak	70 - 95
2	AV + PM + RM	1,0 - 30,5	0,1 - 1,0	very weak - 58	20 - 70
2	EJ	14,5 - 34	0	-	-
1	EJ + PM + RM	21	1,5	55	8
1	PC	104	25	60	40

Concerning frequency of assessment, Agil *et al*. (2008) reported that semen quality increased substantially over one year with repetition of the procedure, and Hermes and Hildebrandt (2012) recommend repeated collections before determining that a male rhinoceros has poor semen quality and limited breeding potential. Interestingly, semen quality does not seem to be affected by age since high quality ejaculates have been consistently obtained from rhinoceros over the advanced age of 30 and up to 42 years of age (Hermes *et al*., 2001a; Hermes *et al*., 2005; Schaffer *et al*., 1998). Regarding social structure, subordinate behaviour has been implied in the high prevalence of reduced semen quality in captive males living with other territorial males (Hermes *et al*., 2005). On the other hand, solitary males have been shown to produce high quality semen, suggesting that female presence is not directly correlated to reproductive fitness (Hermes *et al*., 2005).

9.3. Semen preservation

Studies on White, Black and Indian rhinoceroses showed the motility of high quality sperm suspended in commercial and custom-made extenders and chilled to 4ºC would remain almost constant for 24h, and then gradually reduce to approximately 30% after 72 to 96h. This information indicates that rhinoceros spermatozoa have low sensitivity to chilling, which simplifies the transportation of samples to be used in artificial breeding programmes (Hermes & Hildebrandt, 2012; Hermes *et al*., 2001a; Hermes *et al*., 2005).

As a next step, cryopreservation allows for the use of spermatozoa of distant (captive or wild), reproductively challenged or deceased rhinoceroses in artificial reproduction programmes, therefore increasing the genetic diversity of a population (Hermes & Hildebrandt, 2012; Reid *et al*., 2009). Cryopreservation has been proven an effective method of sperm preservation in White, Black, Indian and Sumatran rhinoceroses (Platz *et al*., 1979; Lubbe, Smith, Bartels & Godke, 1999; Hermes *et al*., 2001a; Stoops *et al*., 2010; O'Brien & Roth, 2000; Roth, Stoops, Robeck & O'Brien, 2015). Furthermore, cryopreserved semen has been successfully used in artificial insemination of White and Indian rhinoceroses, resulting in conception (Hermes *et al*., 2009a; Hermes & Hildebrandt, 2012). Cryopreservation is usually performed in 0,5 ml straws over liquid nitrogen vapour, due to the simplicity of the technique and good post-thaw results. Commercial or custom-made extenders are used for cryopreservation, with glycerol or dimethylsulfoxide (Me_2SO or "DMSO") acting as cryoprotectants (Hermes *et al*., 2005; O'Brien & Roth, 2000; Stoops *et al*., 2010; Reid *et al*., 2009). Since this technique is based on multi-directional heat transference, ice crystals develop at uncontrollable velocity and directions, causing cell damage and death. Therefore, it is essential that the original sperm sample is of high quality, and that after thawing a new assessment of motility, morphology, viability and acrosome integrity is performed (Stoops *et al*., 2010; Reid *et al*., 2009; Hermes & Hildebrandt, 2012). Reported results include post-thaw motilities of 20% to 55% with electroejaculated semen, and up to 63% with epididymal sperm, sometimes representing losses of over 50% the original values (Hermes *et al*., 2001a; Hermes *et al*., 2005; Roth *et al*., 2015; Stoops *et al*., 2010). Nevertheless, all authors report that post-thaw motility of cryopreserved semen is most often enough for assisted reproduction techniques.

Another cryopreservation technology termed directional freezing has only recently been applied to rhinoceros cells. This technique allows for a better control of ice crystal propagation and morphology resulting in less cellular damage, as the sample moves at constant speed through a linear gradient of temperatures. Although it has been proven a superior technique, directional freezing is not extensively used because it requires vulnerable electronics and a higher consumption of liquid nitrogen (Hermes *et al*., 2009a; Reid *et al*., 2009).

9.4. Cellular technologies

Information concerning advanced cellular technologies relevant to male rhinoceroses can be found on page 20 in combination with information relevant for the females.

METHODS AND MATERIALS

1. Ethics statement

The Sabah Rhino Breeding Programme and the present scientific study are in agreement with the WAZA (World Association of Zoos and Aquariums) Code of Ethics and Animal Welfare (11/2003), the German National Act for the Protection of Animals (07/1972, revised on 07/2009) and the Committee of Ethics and Animal Welfare of the IZW. All actions taken in regard to individual animals had the higher aim of assisting in achieving the conservation and survival of the species. Individual animal welfare was compromised under strict limits bearing in mind the extreme conservation status of the species. On the other hand, clinical examinations directed towards the diagnose and treatment of disease are not considered as animal experiments by the German Act for the Protection of Animals, but as mandatory acts of animal welfare. Anesthesia and further medication were employed in agreement with good veterinary practice and respect for animal welfare, to achieve compliance to diagnostic procedures, and reduce or eliminate stress and pain resulting from applied treatments and techniques. In spite of numerous and repeated interventions, none of the animals suffered from negative health effects directly or indirectly attributable to the procedures conducted by the IZW team and described in the present dissertation.

2. Animals

Three females and two males of the Bornean subspecies of Sumatran rhinoceros (*Dicerorhinus sumatrensis harrissoni*) were followed in the present study. All of them originated from Sabah, Malaysian Borneo; and were captured under the national conservation plan for the species, as implemented by SWD and BORA. Examinations from 2005 were conducted at the Rhino Breeding Centre (RBC) located at the Orangutan Rehabilitation Centre in Sepilok, Sandakan. From 2009 onwards the animals were kept at the Borneo Rhino Sanctuary (BRS) in Tabin Wildlife Reserve (TWR), Lahad Datu district, Sabah. In 2015 the 3 surviving individuals (1 male and 2 females) in this study represented one third (33,3%) of the entire captive population of Sumatran rhinoceros, including 50% of the reproductively active females and 33,3% of the mature males. These are also the last known rhinoceroses of Sabah, and likely the last few of Borneo. Names of the animals were used in this document, as opposed to the usual Studbook numbers or codes seen in scientific manuscripts, in order to increase proximity between reader and subject and instil empathy towards the animals and the cause.

2.1. Gelugob

Gelugob (or Gelogob, fig. 3) was a female with estimated birth in 1980. She was captured in June 1994 in Sukau, Kinabatangan. She lived in the RBC in Sepilok, where she mated successfully with Tanjung and one other male (Sidom, deceased in 1997). Gelugob had only one diagnosed pregnancy while in captivity, which was lost after 3 months. She presented no clear signs of previous parturition such as a developed mammary complex. Reports previous to 2005 claim that Gelugob did not cycle regularly. In 2004 Gelugob developed a series of pathological alterations in both eyes, including conjunctivitis and superficial keratitis which evolved to chronic superficial keratitis (*pannus*), with pain and blindness. Swabs for bacteriology revealed the presence of *Staphylococcus sp*. and *Flavobacterium sp.* Treatment was attempted unsuccessfully (Kretzschmar, Sipangkui & Schaffer, 2009). Upon closure of the facilities in Sepilok in 2007, Gelugob was transferred to Lok Kawi Wildlife Park (LKWP) where she lived in apparent poor conditions until 2009 when she was transferred to the sanctuary in TWR. In late 2011, at approximately 30 years of age, Gelugob was considered to be in a post-reproductive phase and therefore no longer able to breed. She was put under governmental care (SWD) and moved back to LKWP in March 2013, where she died tragically 10 months later. Gelugob was reported to be dehydrated and emaciated, having lost almost 100 kg since being transferred. She also presented abrasions and sores on her body, and was severely constipated with over 4,0 kg of sand and stones in her digestive tract (Majawat, 2014). She died of kidney failure in January 2014 with the approximate age of 35 years.

2.2. Puntung

Puntung (or Puntong, fig. 5) is a mature female with estimated birth in 2000. She fell in a pit trap in the Malambabula river area (close to the city of Lahad Datu) in December 2011 after almost 2 years evading capture efforts. She was translocated to TWR by helicopter on Christmas Day, 25th of December 2011. Weighing under 500 kg when captured, Puntung is thought to be the smallest adult rhinoceros ever recorded. This was most likely motivated by the partial absence of her left forelimb, presumably lost in a snare trap at an early age (fig. 6). From this event came also her name “Puntung”, meaning “stump”. When captured, Puntung became immediately tame and adapted easily to life in the breeding facilities. Upon her first clinical examination it was noticed that Puntung’s hymen was ruptured, but no signs of previous parturition were present. Several attempts at natural mating with Tam took place at the BRS in August 2012, December 2012 and October 2013. These were, however, unsuccessful with the male often showing aggressive behaviour and Puntung rejecting him. In 2015 she was thought to be 15 years old.

2.3. Iman

Iman is believed to be the last wild rhinoceros of Sabah (fig. 4). She is a female with estimated birth around 2000. Iman was caught in a pit trap in March 2014 and airlifted from Danum Valley (Lahad Datu district) to TWR some days later. When captured, Iman showed signs of being pregnant and possibly undergoing an abortion, including daily copious bleeding from the vagina. This proved not to be true after urgent specialized examination by the IZW team, which revealed a critically impaired reproductive tract. Iman continued expelling blood, mucus and dead tissue regularly during the study period (discharge reported to be sterile; fig. 9 and 10). Her health status is weaker than Puntung's and a cause for serious concern. She also expresses aggressive behaviour towards the caretakers, which may be motivated by pain or discomfort. In 2015 Iman was believed to be 15 years old.

2.4. Tanjung

Tanjung was a mature male with estimated birth around 1990 (fig. 8). He was captured in July 1993 and translocated to the RBC in Sepilok, where he lived the rest of his life. Tanjung was a reproductively active male, known for the interesting behaviour of masturbating on a tree log in his enclosure. Several mating events were recorded between him and Gelugob, at least once resulting in conception. In spite of having regular erections, Tanjung was believed to have limited breeding potential due to several accounts of artificially induced ejaculation yielding no spermatozoa. In July 2005 Tanjung was also afflicted by conjunctivitis and superficial keratitis of the right eye, resulting in unilateral blindness (Kretzschmar *et al.*, 2009). Tanjung died tragically in August 2006 at the estimated age of 15, victim of a falling tree during a storm.

2.5. Kretam (Tam)

Kretam, commonly and henceforth referred to as Tam (fig. 7), is a mature male with estimated birth in 1990. He was captured in August 2008 after wandering into an oil palm plantation bordering with the Kretam Virgin Jungle Reserve (close to Kulamba Wildlife Reserve, Kinabatangan district). He was visibly injured in his right forelimb and was easily persuaded with leaves to get into a crate. His wound turned out to be caused by a snare trap, and was successfully treated after being translocated to TWR. Tam showed intermittent foot problems, namely fissures in the foot pad, which were successfully managed by the BRS veterinarian. In late 2011 Tam also showed signs of conjunctivitis and superficial keratitis, which were in his case treated successfully. In 2013 it was decided that Tam would be transferred to Cincinnati Zoo to mate with Suci (captive-born female), if no more females were captured in Sabah until the end of 2014. Due to the untimely death of Suci in March 2014 (aged 10, victim of hemochromatosis) this plan was cancelled. In 2015 Tam was believed to be 25 years old.

Figure 3: Gelugob entering a mud wallow at the BRS in 2011. Copyright BORA/IZW.

Figure 4: Iman in her paddock at the BRS in 2014. Copyright BORA.

Figure 5: Puntung after capture in 2014, showing the missing left distal forelimb. Copyright BORA.

Figure 6: Puntung in her paddock at the BRS, showing the missing left distal forelimb. Copyright BORA.

Figure 7: Tam at the BRS in 2011. Copyright BORA/IZW.

Figure 8: Tanjung at the Rhino Breeding Centre in Sepilok, Sandakan. Copyright BORA.

Figure 9: Detail of Iman presenting sanguineous vaginal discharge. Copyright BORA.

Figure 10: Vaginal discharge of blood, mucus and dead tissue presented by Iman. Copyright BORA.

3. Working visits

All ultrasound examinations and advanced assisted reproduction techniques reported in the present study were conducted, unless otherwise stated, by the specialized veterinary team leading the Reproduction Management department of the IZW, namely Prof. Dr. Thomas Hildebrandt, Dr. Frank Göritz and Dr. Robert Hermes. They will be henceforth referred to as "the IZW team". Ultrasound examinations and assisted reproduction procedures included in the present study originated from all animals, in two different time frames. In 2005 the IZW team visited the RBC in Sepilok twice, in April and October, to evaluate the reproductive status of Gelugob and Tanjung. After an interlude the team went back to Sabah to work with the rhinoceroses now at the BRS in TWR: Gelugob, Tam, Puntung and Iman. In the years between 2009 and 2015 the IZW team visited the BRS a total of 15 times: November 2009; January (twice), February and October 2011; February, March, June and November 2012; March and June 2013; April, May and July 2014; and finally April 2015. Another visit took place in October 2015 but the data collected is no longer included in the present study.

4. Restraint and anesthesia

Ultrasonographic assessments and reproductive procedures were performed either by means of operant conditioning or full anesthesia. Positive reinforcement was used in general reproductive examinations by feeding favoured foods (green leafs, bananas and other fruits) while the animal was standing in a restraining chute. In procedures expected to require precision or involve pain and distress, full anesthesia was previously performed. Pharmacological agents used to achieve full anesthesia and subsequent reversal are reported on table 4. Recumbent animals received supplemental oxygen through a nasal cannula at a rate of approximately 15 L/min. Measures taken to prevent compressive trauma of muscles and nerves included the placement of mats below the shoulder and pelvis of recumbent animals, reduced time in sternal *decubitus*, massages to the legs and flanks to improve circulation, and even temporary suspension in strong ropes to allow blood flow to the limbs.

Table 4: Pharmacological agents used to achieve full anesthesia and subsequent reversal in Puntung, Iman and Tam between 2012 and 2015. Values expressed in milligrams (mg) per total body weight of 500 to 650 kg. Total doses were divided in a variable number of supplemental doses throughout the length of the procedure.

	Ketamine	Detomidine	Butorphanol	Midazolam
Induction doses	50 - 100	7,5 - 25	10 - 40	2,5 - 5
Total doses	250 - 1250	11 - 35	35 - 135	5 - 15
		Atipamezole	Naltrexone	
Reversal doses		20 - 60	50 - 200	

5. Ultrasonographic examinations

The internal reproductive organs of both females and males were examined by transrectal ultrasonography as a standardized procedure in all captive rhinoceros species (Stoops *et al.*, 2004; Hermes *et al.*, 2005; fig. 11). Due to their inguinal position, testicles and epididymides were visualized by transcutaneous ultrasonography. An important pre-requisite for good quality imaging in transrectal ultrasonography is thorough cleaning of the rectum. Feces were removed manually and a cleansing enema was performed using a hose with a smooth tip. For transcutaneous imaging in the males, the inguinal area was washed or cleaned of dirt. Lubricant gel was used to overcome anal and rectal tone and to ensure acoustic coupling of the ultrasound waves. When necessary, specialized lubricant such as fertility-friendly and/or sterile gel was employed. Reproductive organs were imaged with a hand-held ultrasound probe in cross and longitudinal sections. Due to small size of the animals, the use of extensions was not necessary. During the examination some of the most important structures were measured as still images using standard calibrated software included in the ultrasound units. Video sequences and still images of all ultrasound examinations were recorded for retrospective analysis. In 2005 the ultrasonography unit used by the team was the SonoSite 180 Plus (SonoSite GmbH, Frankfurt a.M., Germany) equipped with low frequency convex and micro-convex probes (2-4 MHz) and high frequency linear transducers (5-10 MHz). Videos of the ultrasound examinations were recorded using a Sony Watchman GV-D900E coupled with Sony DVM60 Mini DV Cassette, and analysed retrospectively using DV Gate Still software (all from Sony, Germany). From 2009 onwards the IZW team used a Voluson-i ultrasound system equipped with broadband 2D linear (5-13 MHz) transducers; convex (1-5 and 2-8 MHz), micro-convex (3-9 MHz) and linear (6-18 MHz) volume transducers (GE Healthcare, Berlin, Germany). Files were recorded and analysed on the ultrasound unit using the built-in software.

Figure 11: Transrectal ultrasound examination in Puntung using operant conditioning. In the picture Prof. Dr. Thomas Hildebrandt, Dr. Robert Hermes and Dr. Zahari Zainuddin. Copyright BORA/IZW.

6. Analysis of ultrasonographic examinations

For the present study, recorded ultrasonographic examinations were analysed by the author. In order to increase accuracy and reduce variability, the following set of measures was employed:

1) specific knowledge was transmitted by the specialists to the less experienced researcher;

2) measurements reported by the specialist team during actual examination were initially rejected in order to reduce inter-operator variability;

3) each ultrasonographic examination was carefully and systematically analysed at least twice in different time frames;

4) if significant doubts persisted the specialist team would be consulted;

5) measurements and notes available from the original examinations were finally used for comparison, as a method of self-evaluation.

A description of the systematic analysis undertaken follows.

6.1. Females

Examined organs of the females were the urinary bladder, vagina, cervix, uterine body, uterine horns, uterine tubes and ovaries. Notes were made on structure and sonographic appearance of each organ, including abnormal features like masses and cysts. A total of 20 measurements were systematically investigated and obtained whenever possible: combined thickness of the vagina; smallest diameter, largest diameter and length of the cervix; total diameter and combined endometrial thickness of the uterine body; total diameter and combined endometrial thickness of both uterine horns; length, height and width of both ovaries; length and height or diameter of all identified follicles and *corpora lutea*.

6.2. Males

Studied organs of the males were the pelvic and prostatic urethra, urinary bladder, bulbourethral, prostate and seminal glands, testicles and epididymides. Notes were taken on structure and sonographic appearance of each organ whenever possible. A total of 38 measurements were systematically investigated and obtained whenever possible: total diameter and mucosal thickness of the pelvic urethra; total diameter and mucosal thickness of the prostatic urethra; length, height and width of both bulbourethral glands; length, height and width of both prostate lobes; length, height and width of both seminal glands; length, height and width of both testicles; thickness of the *mediastinum* of both testicles; length and width of the tail of both epididymides; width and height of the body of both epididymides.

7. Ultrasonography validation

The accuracy of methods used in the present study was tested by the IZW team and published in Hermes *et al.* 2006a. The authors found that measurements of rhinoceros reproductive organs and pathological features taken from ultrasound examinations were very highly correlated with measurements obtained directly from *post-mortem* preparations ($n=38$; $r^2=0,9$ with $p<0,001$).

8. Statistical analysis

Given the descriptive nature of the work at hand, only descriptive statistics were employed. For each analysed parameter the author presents the mean value of the sample ("mean" or "average"), the standard error of the mean ("SEM"), maximum value, minimum value and sample size ("n"). Standard error of the sample mean is presented instead of standard deviation of the sample ("SD") in order to quantify the precision of the calculated mean. The author recalls that standard error of the mean is dependent on both standard deviation and sample size, as dictated by the simple relation $SEM=SD/\sqrt{(n)}$. Occasionally when the sample is larger, standard deviation may also be presented.

9. Assisted reproduction procedures

All actions and procedures aiming at increasing the reproductive potential of the animals under study were analysed by the author in regard to method, outcome and significance. These actions and procedures were developed and/or applied by the specialized veterinary team of the IZW and associate specialists, and include hormonal treatments, artificial insemination, oocyte collection and *in vitro* techniques for the females; and semen collection, evaluation and preservation in reference to the males. Also included in this section are the more clinical procedures aiming at the removal of endometrial cysts and one hydrosalpinx.

10. Endocrinology

Serum progesterone values between 2011 and 2015 were provided by Dr. Zainal Zahari Zainuddin, chief veterinarian at the BRS. They were obtained from serum samples systematically collected by Dr. Zahari Zainuddin and analysed at a local laboratory in Sabah (PathLab, Sandakan) through direct chemiluminescence immunoassay. Although the methodology was not validated for this species and total values may be inaccurate, the method was constant and observation of cyclical changes in concentration is thought to be sufficient for the analysis at hand.

RESULTS

1. Working visits and assisted reproduction procedures

A total of 17 working visits between April 2005 and April 2015 were included in the present study. Actions and procedures conducted during these visits and analysed in the present study include 56 reproductive assessments by ultrasonography, 10 hormonal treatments, 1 artificial insemination (AI), 5 oocyte collections, 3 intracytoplasmic sperm injections (ICSI), 4 techniques for the removal of endometrial cysts, 1 hydrosalpinx aspiration and 8 semen collections by electroejaculation.

2. Ultrasonographic examinations

A total of 56 reproductive assessments done by means of ultrasonography were analysed, in which 16 were from Gelugob, 16 from Puntung, 6 from Iman, 5 from Tanjung and 13 from Tam. This correlates to approximately 950 ultrasound files (videos and still images) with an average 16,40 recorded files per ultrasound examination (SD=8,50 and SEM=1,14).
Average time between first and last recording of an ultrasound examination during general reproductive assessments performed after 2009 can be consulted on table 5. In Tam's case, recorded examinations were often incomplete focusing only on testicles and epididymides or only on accessory sex glands.

Table 5: Time between first and last recorded ultrasound files, in minutes rounded to the first decimal place except when accurate to the units. Data refers only to examinations performed between 2009 and 2015.

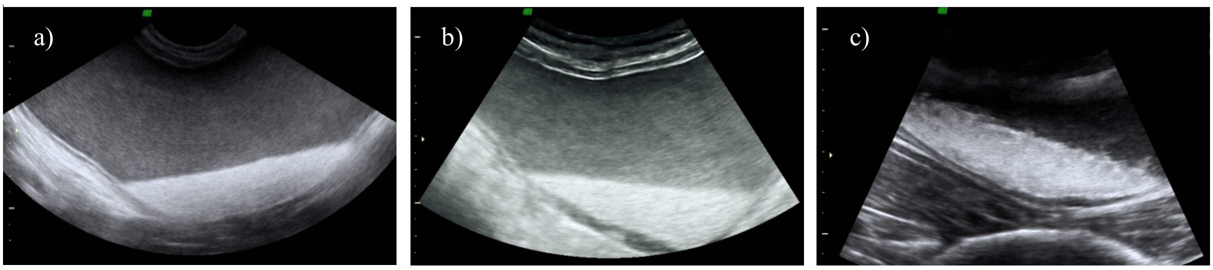
	Gelugob	Puntung	Iman	Tam
Mean	18,5	12,9	13	9,1
SEM	2,4	1,1	2,9	1,7
Minimum	8	6	5	4
Maximum	27	22	23	18
Sample size	8	13	5	8

3. Ultrasonographic findings in female rhinoceroses

3.1. Urinary bladder

When performing transrectal ultrasonography in a female, the first structure to be located and examined is the *vesica urinaria*, or urinary bladder. In a longitudinal view, the urinary bladder appears as a teardrop-shaped structure with a thin wall and fluid content. In all examined animals (both females and males) urine appeared echogenic due to large amounts of particles scattered through the bladder lumen, sometimes giving it the appearance of a starry sky or producing swirling echo patterns. Settled sediment was also observed on some occasions, in all individuals (fig. 12). Measurements obtained from the observed deposits were surface length (n=6), depth (n=6) and width (n=2). Deposits of urinary sediment in the bladder ranged from 5,00 to 8,80 cm in surface length; 1,00 to 3,90 cm in depth; and 2,85 to 4,40 cm in width. Approximate volume of the deposits was calculated using half the volume of an ellipsoid* with its major axis corresponding to surface length, minor axis corresponding to width, and vertical axis corresponding to two times the depth of the deposits. When width was not possible to assess, a value corresponding to twice the depth of the deposit was used. Results found that approximate volumes of deposited urinary sediment ranged from 13 cm^3 to 29 cm^3 after 2009, possibly reaching over 70 cm^3 in one individual in 2005.

Figure 12: Sonograms of the urinary bladder showing hyperechogenic urine and settled sediment, in a) Puntung, b) Gelugob and c) Tam. Scale of 6,0 cm for a), 6,5 cm for b) and 5,5 cm for c). Copyright IZW.

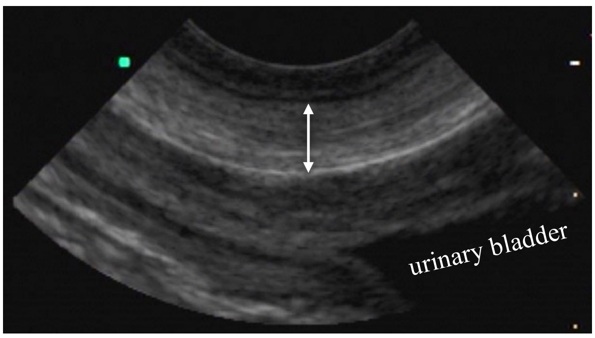


The inability to control the volume of urine enclosed in the bladder at the time of examination makes it hard to take conclusions on wall thickness and layering. Nevertheless, no alterations of the epithelial layer suggesting possible inflammation or neoplasia were noted, except on one occasion. In July 2014 Iman presented two well-defined structures in the bladder wall, which resembled mucosal folds protruding to the lumen. The structures measured 0,41 and 0,42 cm in height with 0,14 and 0,22 cm in thickness, and were parallel with just a few millimetres between them. The bladder was moderately full and no other abnormalities were observed. On the following examination these structures were no longer visible.

3.2. Vagina

The *vagina* can be identified between the urinary bladder and the rectal wall, caudally to the cervix. In a longitudinal view it appears as two adjacent layers of soft tissue (fig. 13). Measurements were possible to obtain on 9 examinations from Gelugob and Puntung, with an average total thickness of 0,65 cm. Specific average values for each animal can be

Figure 13: Sonogram of Gelugob's vagina (arrow). Scale of 2,0 cm. Copyright IZW.



* volume of an ellipsoid with a, b and c as the major, minor and vertical axes = (1/6)πabc

seen on table 6. The lumen of the vagina was never patent in the sonograms of these females. No alterations of normal structure or echogenicity indicating inflammatory or neoplastic changes were observed. In regard to Iman, it was only possible to evaluate her vagina in a transverse scan on one occasion. The sonogram revealed a total thickness of 1,27 cm with slightly hypoechoic mucosa and presence of anechogenic fluid in the lumen.

Table 6: Quantitative description of obtained data regarding thickness of the vagina in 2 females, in centimetres.

	General	Gelugob	Puntung
Mean	0,65	0,67	0,57
SEM	0,04	0,05	0,03
Minimum	0,50	0,50	0,54
Maximum	0,90	0,90	0,60
Sample size	9	7	2

3.3. Cervix

The *cervix uteri* can be identified dorsally to the urinary bladder and followed cranially over the pelvic brim. In a longitudinal scan the cervix appears as alternating hyperechoic and hypoechoic contours, revealing the existence of tightly interlocked folds and a tortuous canal (fig. 14). Analysed sonograms indicate the cervix of Sumatran rhinoceroses has a long ovoid shape, with its diameter increasing gradually from the external ostium, reaching a maximum thickness and then decreasing before giving origin to the uterine body (fig. 15). The diameter of the cervix in its origin was only possible to measure consistently in Gelugob, revealing an average value of 1,90 cm (table 7). On the other hand, maximum thickness of the cervix was measured on 17 different exams in all females, with a general average value of 3,91 cm (table 7). Specific average values for maximum diameter in each female can be seen on table 8. Cervical length was often impossible to measure due to the field of view, but when possible estimated lengths varied between 8,0 and 9,0 cm in Gelugob and Puntung (table 7).

Figure 14: Sonogram of Gelugob's cervix showing interlocked folds. Scale of 5,0 cm. Copyright IZW.

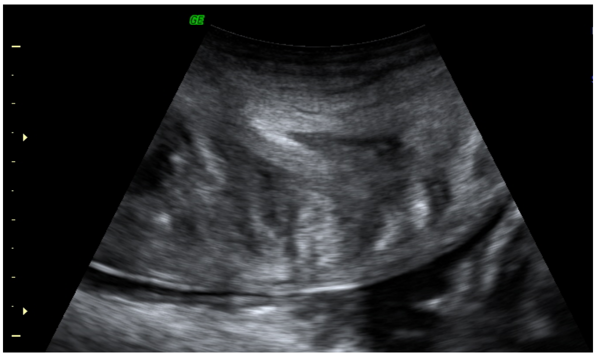


Figure 15: Sonograms of Puntung's cervix showing a long ovoid shape. From right to left it is possible to see the cervix a) increasing, b) reaching a maximum diameter, and c) decreasing. Scale of 4,0 cm. Copyright IZW.

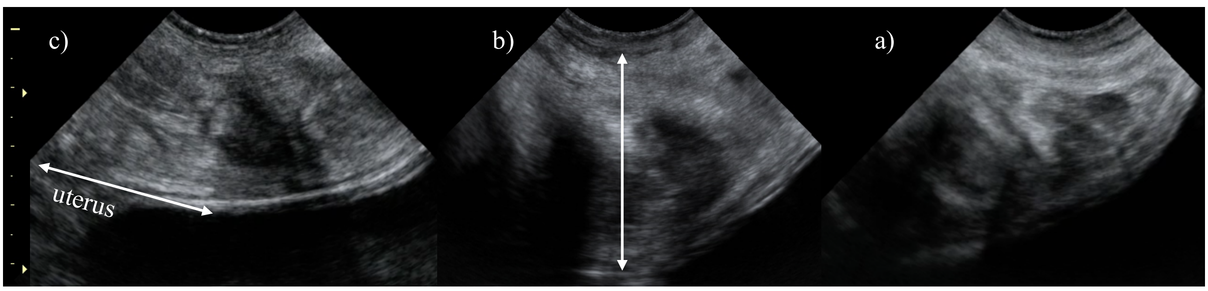


Table 7: Quantitative description of obtained data regarding the cervix of all females, in centimetres.

	Initial diameter	Maximum diameter	Length
Mean	1,90	3,91	8,67
SEM	0,08	0,22	0,17
Minimum	1,65	2,62	8,00
Maximum	2,20	5,40	9,00
Sample size	6	17	6

Table 8: Quantitative description of obtained data on maximum diameter of the cervix of each female, in cm.

	Gelugob	Puntung	Iman
Mean	3,53	3,44	5,25
SEM	0,21	0,31	0,09
Minimum	2,67	2,62	5,00
Maximum	4,50	4,00	5,40
Sample size	9	4	4

Analysed sonograms revealed cervices with well-preserved structure and echogenic properties in all females. Only 3 anomalies were identified. In November 2009 a mass was observed in the muscular wall of Gelugob's cervix. The mass was bean-shaped with 1,68 x 0,87 cm, presented a hypoechoic border and protruded outwards. The other identified anomalies were a single small cyst in the mucosa of both Puntung's and Iman's cervices. Puntung's cervical cyst was detected in May 2014 in proximity to the external ostium, and measured 0,25 cm in diameter. Iman's cervical cyst was observed during her first examination in April 2014 closer to the internal ostium, and measured 0,30 cm in diameter.

3.4. Body of uterus

The *corpus uteri* is located cranially to the cervix and consists in a remarkably short tubular organ that later divides in two uterine horns. Several layers become visible in a transverse longitudinal scan of the uterine body: the thin hyperechogenic serosa as the outward layer; a thick layer of myometrium in the middle; a hypoechoic line reflecting the vascular network in the interface between inner myometrium and basal endometrium; the more or less hypoechogenic layer of endometrium; and the central hyperechoic line resulting from the junction of the two functional layers of the endometrium (fig. 16 and 17).

Figure 16: Sonogram of Gelugob's uterus (big arrow) showing an inactive endometrium (small arrow). Scale of 2,0 cm. Copyright IZW.

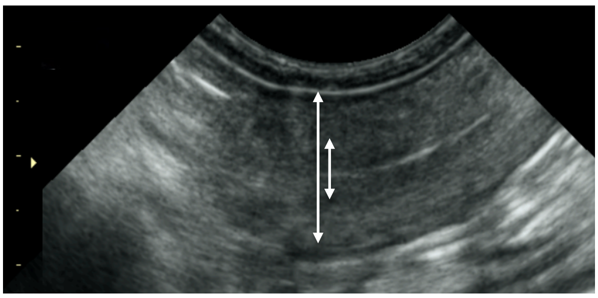
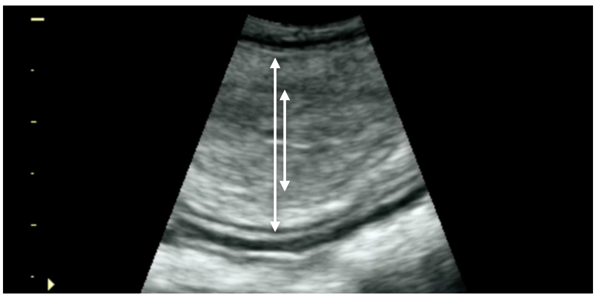


Figure 17: Sonogram of Puntung's uterus (big arrow) showing a more developed endometrium (small arrow). Scale of 2,5 cm. Copyright IZW.



The lumen of the uterus was never observed as an anechoic band (suggesting the presence of fluid) while normal structure was maintained. Length of the uterine body was not possible to determine accurately due to scanning method and field of view, except on one occasion in Puntung when it appeared to measure approximately 4,00 cm. Total thickness of the uterine body was evaluated on 25 examinations of all females, revealing a general average value of 2,56 cm in diameter (table 9). Specific average values for each female can be seen on table 10. The endometrium was measured on 24 examinations as a double layer, between both hypoechoic lines that separate myometrium and endometrium. General average value for endometrium double-layer thickness was 1,36 cm (table 9), and specific average values for each female can be consulted on table 10. When both measurements were obtained, a ratio between double-layer endometrium (a) and total thickness of the uterus (b) was calculated and expressed in percentage ([a x 100] / b). Results varied between 40,0% and 70,9% of endometrium in the total thickness of the uterus, with the general average reaching 53,7% (table 9). Specific average values for each female are presented on table 10.

Table 9: Quantitative description of obtained data on the uterine body of all females, in cm and percentage (%).

	Total diameter	Endometrium	Percentage
Mean	2,56	1,36	53,7
SEM	0,62	0,41	9,1
Minimum	1,73	0,73	40,0
Maximum	4,05	2,05	70,9
Sample size	25	24	24

Table 10: Quantitative description of obtained data on the uterine body of each female, in cm and percentage (%).

	Gelugob			Puntung			Iman		
	Total	End.	%	Total	End.	%	Total	End.	%
Mean	2,17	1,02	46,4	2,67	1,61	60,6	4,03	2,05	50,6
SEM	0,11	0,08	1,7	0,12	0,07	1,7	0,02	-	-
Minimum	1,73	0,73	40,0	1,99	1,18	48,8	4,00	-	-
Maximum	2,70	1,50	55,6	3,38	2,02	70,9	4,05	-	-
Sample size	11	11	11	12	12	12	2	1	1

Total thickness and double-layer endometrial thickness were often very hard to assess due to poor condition of the uterus of Puntung and especially Iman. Measurements were obtained from areas with higher preservation of normal anatomical structure, and were not performed when no acceptable areas were found. Such was often the case of Iman, hence the lack of data regarding her uterus (table 10).

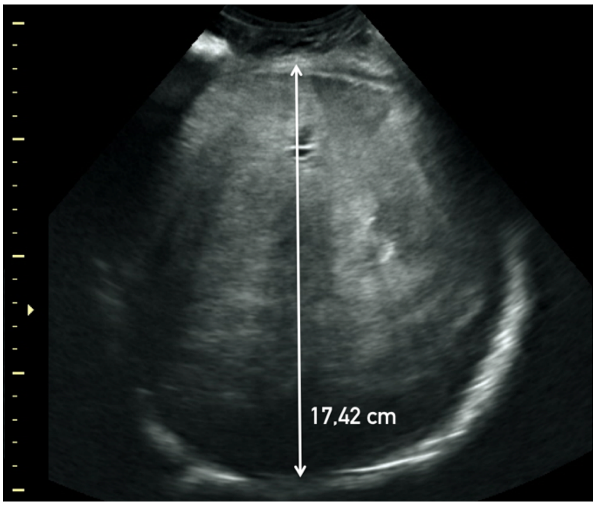
Relevant differences were encountered when analysing the uterus of each female separately. Gelugob presented, especially after 2009, an inactive uterus with a flat endometrium (fig. 16). It is possible to see a decreasing trend, through time, in percentage of endometrium to total thickness of the uterus: while in 2005 the average value was 52,7% and in 2009 52,9%, in 2011 it went down to 43,2%, and in 2012 to 40,0%. Gelugob presented one small endometrial cyst in the uterine body which suffered a reduction in diameter between 0,50 cm upon detection in 2005 to 0,25 cm in 2009 and 0,15 cm in 2011. Later in 2011 (following an hormonal treatment) the original cyst increased in dimension and a second cyst measuring between 0,15 and 0,30 cm was detected. Gelugob's endometrium did not show any other alteration, namely images consistent with endometrial fibrosis.

In what concerns Puntung, the first examination after capture (early 2012) revealed a severely affected uterus with dozens of cysts throughout the endometrium (fig. 18). The hyperechoic middle line seen in the uterus was irregular and often not visible due to pathological changes, and presumably indicating the cysts were protruding to the lumen. In the uterine body, multiple endometrial cysts could be seen measuring up to 1,10 cm in 2012 and 1,40 cm in 2013 and 2014. In June 2012 a clear improvement in number and dimension of the cysts was observed. However, in November 2012 the pathological changes were exacerbated once again. Similar findings were observed in March 2013 and July 2014, with the uterus revealing a worse condition than on previous examinations. One other pathological structure identified on Puntung's uterine body in early 2012 was a mass close to the internal bifurcation of the uterine horns. The ultrasonographic image of this structure was hyperechoic, homogeneous with well-defined boundaries and apparently not protruding. Although irregular in shape, the hyperechogenic mass maintained a diameter of approximately 0,50 or 0,60 cm through time.

Figure 18: Sonogram of Puntung's uterus showing numerous large endometrial cysts. Scale of 4,0 cm. Copyright IZW.

The condition of Iman's uterus proved to be even more alarming. Upon capture (April 2014) she presented multiple uterine masses with varying size and echogenicity (fig. 19). Approximately 10 masses measured over 4,00 cm in diameter, with the largest one reaching 17,42 cm. In terms of echogenicity, the masses showed some heterogeneity between and within each other, with some being more hypoechogenic than others, and some displaying hyperechoic patches suggesting tissue necrosis (fig. 19). Some of the largest masses exhibited hypoechoic tube-like cavities, which combined with colour Doppler signals point to the existence of structured internal blood supply. The mass with 17,42 cm presented the broadest hypoechoic elongated cavity, localized in the centre of the tumour and reaching 1,18 cm in cross section. Multiple endometrial cysts were also identified in Iman's uterine body, measuring up to 1,62 cm in diameter. Contrarily to Puntung, normal anatomical structure of Iman's uterus was very hard to identify on recorded examinations, as was locating the pathological structures. On some occasions anechoic areas were observed surrounding the masses, suggesting the presence of fluid within the uterus.

Figure 19: Sonograms of Iman's largest uterine mass. Maximum diameter marked with an arrow. Note the differences in echogenicity within the mass. Scale of 20,0 cm on the left and 16,0 cm on the right. Copyright IZW.



3.5. Uterine horns

The *cornua uteri* appear internally after the end of the uterine body, and run side by side united by a muscular layer. After a few centimetres the horns separate externally and proceed in opposing directions. The uterine horns were most frequently examined in longitudinal cross section, and several layers could be observed similarly to the uterine body. The lumen of the uterine horns was never visualized as an anechoic band while normal structure was maintained. Length of the uterine horns was not possible to assess through ultrasonography. Further measurements of the uterine horns were obtained from all females on 29 examinations. Maximum diameter was evaluated a total of 35 times, in a combination of both (n=12), only one (n=16) or zero (n=7) sides identified between left and right. General average value for all females was 1,52 cm without distinction from side (table 11), and specific average values for each female can be seen on table 12. The endometrium was also measured as a double layer on all opportunities with the general average value reaching 0,78 cm (table 11). Specific average values for each female are shown on table 12. A ratio between double-layer endometrium and total thickness of the uterine horns was calculated and expressed in percentage as done for the uterine body. Results varied between 28,6% and 73,8% with the general average falling on 50,1% (table 11). Specific average values for each female are presented on table 12.

Table 11: Quantitative description of obtained data regarding the uterine horns of all females, in centimetres and percentage (%), without distinction between left and right sides.

	Total diameter	Endometrium	Percentage
Mean	1,52	0,78	50,1
SEM	0,06	0,05	2,0
Minimum	0,93	0,34	28,6
Maximum	2,27	1,55	73,8
Sample size	35	35	35

Table 12: Quantitative description of obtained data on the uterine horns of each female, in centimetres and percentage (%), without distinction between left and right sides.

	Gelugob			Puntung			Iman		
	Total	End.	%	Total	End.	%	Total	End.	%
Mean	1,39	0,57	40,3	1,67	1,01	60,1	1,57	0,91	56,3
SEM	0,07	0,04	1,4	0,10	0,07	1,6	0,16	0,18	7,0
Minimum	0,93	0,34	28,6	0,97	0,56	47,0	1,13	0,50	44,2
Maximum	1,80	0,80	50,0	2,27	1,55	68,3	1,83	1,35	73,8
Sample size	17	17	17	14	14	14	4	4	4

As in the uterine body, measurements of the horns were sometimes hard to assess due to pathological changes, and were only obtained from areas with higher preservation of normal anatomical structure. Pathological changes observed in the uterine horns resembled those identified in the uterine body of each female. Gelugob showed a flat uniform endometrium with one or two cysts measuring approximately 0,20 cm after 2011. Images compatible with endometrial fibrosis were not obtained.

Puntung in her turn presented multiple small to medium-sized cysts (fig. 20), which improved in June 2012 only to get worse in November 2012. Worst condition of the uterine horns was observed in July 2014, when uncountable cysts occupied the endometrium. Particularly important were the large cysts discernible at the cranial end of each uterine horn, apparently occluding the uterine lumen (fig. 21). Through time they measured between 0,70 and 1,40 cm on the left side, and between 0,80 and 1,60 cm on the right side. These pathological structures showed a general tendency to increase in size starting with an average diameter of 0,80 cm in 2012, and reaching approximately 1,50 cm in 2013 and 2014. When general condition of

Figure 20: Sonogram of Puntung's uterine horn showing numerous large endometrial cysts. Scale of 3,5 cm. Copyright IZW.

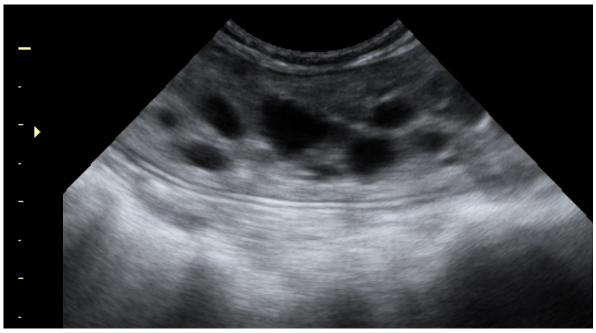
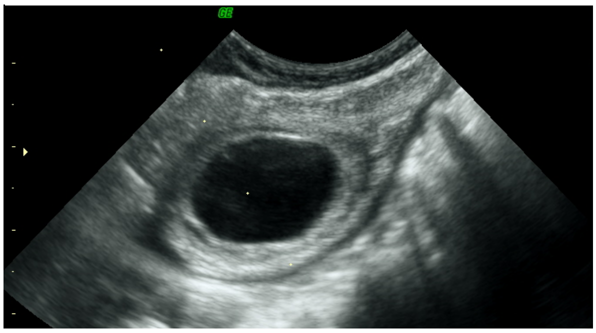


Figure 21: Sonogram of Puntung's uterus showing a large cyst in one of the horns. Scale of 3,0 cm. Copyright IZW.

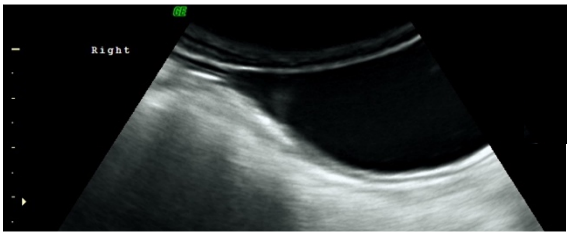


the uterus improved in June 2012, the large cranial cysts did not seem to be affected. In March 2013 a procedure for the specific removal of these structures was conducted, with good immediate results. However, in June 2013 they had returned and at least in May 2014 had achieved or exceeded their previous dimensions. The right uterine horn also presented a large cyst with denser content, which measured between 0,50 and 1,10 cm in 2012. Images compatible with endometrial fibrosis were not observed. In regard to Iman, the condition of the uterine horns mimicked that of the uterine body, with masses as previously described and endometrial cysts measuring up to 1,20 cm.

3.6. Uterine tubes

During Iman's first examination, a large anechoic structure was observed next to one of the uterine horns. This pathological structure was later identified as the right *tuba uterina* or *salpinx*, dilated with fluid (hydrosalpinx) until an average of 4,86 by 3,10 cm (n=5, fig. 22).

Figure 22: Sonogram of Iman's right uterine tube dilated with fluid. Scale of 3,5 cm. Copyright IZW.



3.7. Ovaries

When visualized by ultrasonography the *ovaria* resembled elongated ellipsoids, although it was often difficult to define their limits. Large ovarian structures seemed to modify the size and shape of each ovary as a whole, rather than protrude on the surface. The outer hypoechoic cortex and the inner hyperechoic medulla were possible to discern on occasion, especially in inactive ovaries. When functional structures were present no exact organization was apparent (fig. 23 and 24). The ovaries were measured most often from 2D longitudinal scans, in length and height. Whenever transverse plans or 3D scans were performed, width was obtained as the third dimension. Average dimensions of Gelugob's ovaries on 13 different examinations were 3,70 x 1,30 x 2,07 cm on the right side and 3,82 x 1,42 x 2,08 cm on the left side. In Puntung's case, average dimensions on 14 examinations were 5,28 x 2,30 cm for the right ovary and 4,70 x 2,22 cm for the left ovary. Maximum recorded length for Puntung's ovaries was

Figure 23: Sonogram of Puntung's left ovary showing a large follicle and a central clot CL. Scale of 3,0 cm. Copyright IZW.

Figure 24: Sonogram of Puntung's left ovary showing a large dense CL. Scale of 3,0 cm. Copyright IZW.

approximately 9,00 cm by the end of 2012. Iman's ovaries were only measured on 3 occasions, resulting in a length of 10,16 cm for the left ovary, and an average height of 3,52 cm for the left and 2,78 cm for the right. Further information on right and left ovaries of each female can be found on tables 13 and 14.

Table 13: Quantitative description of obtained data regarding the right ovary of each female. Measurements obtained are length (L), height (H) and width (W), in centimetres.

	Gelugob			Puntung			Iman		
	L	H	W	L	H	W	L	H	W
Mean	3,70	1,30	2,07	5,28	2,30	-	-	2,78	-
SEM	0,25	0,06	0,11	0,81	0,11	-	-	0,23	-
Minimum	2,06	1,00	1,80	3,90	1,76	-	-	2,55	-
Maximum	5,00	1,60	2,27	9,00	2,84	-	-	3,00	-
Sample size	12	11	4	6	12	0	0	2	0

Table 14: Quantitative description of obtained data regarding the left ovary of each female. Measurements obtained are length (L), height (H) and width (W), in centimetres.

	Gelugob			Puntung			Iman		
	L	H	W	L	H	W	L	H	W
Average	3,82	1,42	2,08	4,70	2,22	-	10,16	3,52	-
SEM	0,20	0,08	0,08	0,12	0,14	-	-	0,69	-
Minimum	2,46	1,00	1,80	4,50	1,46	-	-	2,51	-
Maximum	4,70	2,00	2,40	5,00	2,94	-	-	4,85	-
Sample size	13	13	6	4	12	0	1	3	0

Different ovarian characteristics were seen in each female, reflecting different reproductive conditions and metabolic statuses. Regarding Gelugob, in 2005 she presented 2 CLs measuring between 1,00 and 1,40 cm in the left ovary, and several small 0,25 cm anechoic structures in the right ovary. When she was evaluated again in 2009, the left ovary was inactive but in the right ovary the anechoic structures persisted (fig. 26). Upon further examination it became clear the anechoic images corresponded to transverse views of enlarged blood vessels from the vascular network of the ovarian stroma. Hormonal treatments to stimulate follicular growth were employed in 2011 but failed in achieving their purpose (see ahead page 55). Gelugob's left ovary exhibited since 2009 small central hyperechoic spots suggestive of fibrosis (fig. 25). Following the hormonal treatments, a few hypoechoic cavities appeared also in the left ovary.

Figure 25: Sonogram of Gelugob's left ovary in 2011. Scale of 2,0 cm. Copyright IZW.

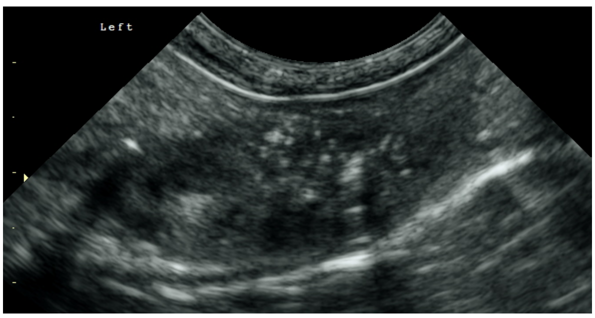
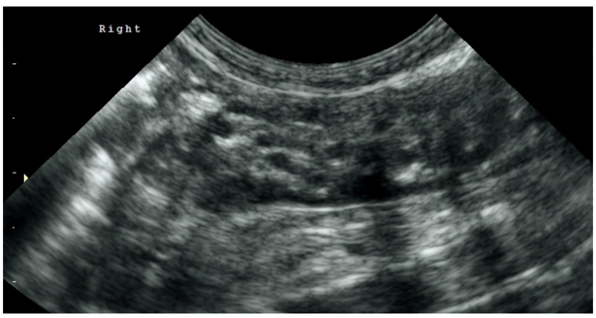


Figure 26: Sonogram of Gelugob's right ovary in 2011. Scale of 2,0 cm. Copyright IZW.



Quite the reverse situation, both Puntung and Iman presented functional structures on all examinations. A description of the findings follows.

3.7.1. Follicles

Puntung's ovaries were studied on 14 different examination days and were only void of follicles in the first assessment after capture. Follicles were observed in a number between 1 and 4 on both ovaries at one time, with a maximum of 3 per ovary. Frequency of observation of each number of follicles is reported on table 15, revealing that Puntung most often presented more than 1 follicle. In total, 35 follicles were analysed measuring between 0,23 cm and 2,32 cm in largest diameter. Distribution of studied follicles by dimension can be seen on table 16, which reveals the great majority of observed follicles fell below 2,0 cm in diameter.

Table 15: Total number and frequency of examination days in regard to observed number of follicles. Data concerning Puntung.

Number of follicles	n = 14 examination days	
1	1	7,1 %
2	4	28,6 %
3	6	42,9 %
4	2	14,3 %

Table 16: Total number and frequency of studied follicles falling in each interval of diameters (in centimetres). Data regarding Puntung.

Dimension of follicles	n = 35 studied follicles	
[0; 1[	18	51,4 %
[1; 2[	15	42,9 %
[2; 3[	2	5,7 %

Iman's ovaries were only studied on 3 different examinations days. In each of those days she presented a total of 4, 5 and 8 follicles on both ovaries, with a maximum number of 6 per ovary at one time (table 17). A total of 17 follicles was analysed, measuring between 0,25 and 2,38 cm in largest diameter. Distribution of studied follicles by dimension can be seen on table 18, revealing the great majority of observed follicles fell below 2,0 cm in diameter.

Table 17: Total number and frequency of examination days in regard to total number of follicles. Data concerning Iman.

Number of follicles	n = 3 examination days	
4	1	33,3 %
5	1	33,3 %
8	1	33,3 %

Table 18: Total number and frequency of studied follicles falling in each interval of diameters (in centimetres). Data regarding Iman.

Dimension of follicles	n = 17 studied follicles	
[0; 1[	4	23,5 %
[1; 2[	10	58,8 %
[2; 3[	3	17,6 %

In both females, follicle shape was dependent on the amount of functional structures in the same ovary, with visible variations from round to cylindrical or irregular shape (fig. 23). Largest diameter of the follicles without suffering ecographic alterations was approximately 2,30 cm. Twice it was possible to measure the growth rate of dominant follicles in Puntung. During a natural cycle in June 2012 one follicle grew from 1,05 to 1,22 cm in 16h while on the other ovary 2 follicles measuring 1,15 and 0,80 cm stagnated or decreased (next to the decreasing largest dense CL), which indicates a growth rate of approximately 0,01 cm/h or 0,25 cm/day for the selected dominant follicle. In June 2013, also during a natural cycle, one follicle grew from 1,22 to 1,83 cm in 72h while another grew from 1,28 to 1,48 cm (and then regressed), indicating approximate growth rates of 0,20 cm/day for the selected dominant follicle and 0,07 cm/day for the subordinate follicle. These two examples also show that selection of the dominant follicle may occur between approximately 1,00 and 1,20 cm; and that other factors besides growth rate play a role, since the largest follicles sometimes do not become the dominant follicles. Finally, ovulation was not evident in any of the examinations performed.

3.7.2. Luteal structures

Puntung's ovaries also presented luteal structures in all examinations except once in 2014 after a long hormonal treatment. Maximum number of CLs at one time in Puntung was 4 per ovary and 6 in both ovaries. Sometimes all luteal structures were located in the same ovary, and twice this was observed with 4 CLs. Frequency of observation of each number of CLs is reported on table 19, making it clear that most often Puntung presented more than one CL. In total 41 CLs were analysed, measuring between 0,60 and 3,00 cm in largest diameter. Distribution of studied CLs by dimension can be seen on table 20.

Table 19: Total number and frequency of examination days in regard to total number of luteal structures. Data concerning Puntung.

Number of luteal structures	n = 14 examination days	
1	1	7,1 %
2	3	21,4 %
3	4	28,6 %
4	4	28,6 %
6	1	7,1 %

Table 20: Total number and frequency of studied luteal structures falling in each interval of diameters (in centimetres). Data regarding Puntung.

Dimension of luteal structures	n = 41 studied luteal structures	
[0; 1[	10	24,4 %
[1; 2[	21	51,2 %
[2; 3[	9	22,0 %
[3; 4[	1	2,4 %

In each of the 3 examination days, Iman presented 2, 4 and 8 CLs on both ovaries, with a maximum of 5 structures per ovary at one time (table 21). Therefore, 14 CLs were analysed, measuring between 0,52 and 1,88 cm in largest diameter. Distribution of studied luteal structures by dimension can be seen on table 22.

Table 21: Total number and frequency of examination days in regard to total number of luteal structures. Data concerning Iman.

Number of luteal structures	n = 3 examination days	
2	1	33,3 %
4	1	33,3 %
8	1	33,3 %

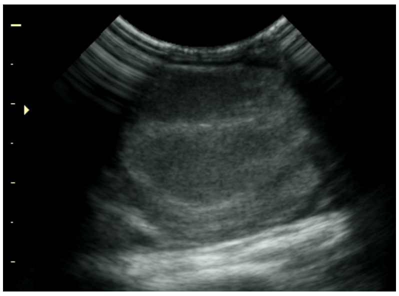
Table 22: Total number and frequency of studied luteal structures falling in each interval of diameters (in centimetres). Data regarding Iman.

Dimension of luteal structures	n = 14 studied luteal structures	
[0;1[	7	50,0 %
[1; 2[	7	50,0 %

Comparing number of follicles and number of CLs on both ovaries at each examination day, often the number of CLs is higher. In Puntung this was observed 53,8% (7) of times, while on 15,4% (2) of days the number was equal and on 30,8% (4) lower. On the other hand, in Iman this was observed once (33,3%) while twice the number of follicles was higher.

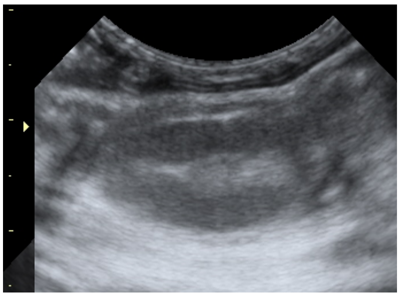
Regarding morphology, 3 different types of luteal structures were observed. The most common form was a dense *corpus luteum*, moderately echogenic with an elongated hyperechoic centre and homogeneous parenchyma (fig. 27). This form accounted for 63,4% of Puntung's studied luteal structures, 92,9% of Iman's and all of Gelugob's (table 23). When all dense CLs were analysed together, largest diameter ranged from 0,52 to 2,82 cm (table 23). On 2 occasions it was possible to study the structural regression of the largest dense CL. In June 2012 the largest dense CL decreased from 2,30 to 1,98 cm within 16h while a dominant follicle was growing on the other ovary, which indicates a shrinking rate of approximately 0,02 cm/h or 0,50 cm/day. In June 2013 the largest dense CL decreased from 2,82 to 2,40 cm in 72h while 2 follicles grew in the same ovary, indicating a shrinking rate of approximately 0,14 cm/day.

Figure 27: Sonogram of a dense *corpus luteum*. Scale of 3,0 cm. Copyright IZW.



The second most frequent form of luteal structure was one exhibiting a central clot. This structure was similar to the dense CL with homogeneous parenchyma and an elongated hyperechoic centre, which in this case was interrupted by a hypoechoic central clot (fig. 28). This form was responsible for 26,8% of Puntung's luteal structures and 7,1% of Iman's (table 23). When studied together, central clot CLs measured between 0,60 and 1,88 cm in largest diameter (table 23).

Figure 28: Sonogram of a central clot *corpus luteum*. Scale of 2,5 cm. Copyright IZW.



Lastly, on 4 occasions large presumably luteinized structures were observed in Puntung, with thick echogenic walls and a large anechoic centre with numerous fibrous bands organized in a spiderweb-like structure (fig. 29). In order to avoid confusion with previous definitions of terms such as *corpora hemorrhagica,* at present stage these are termed "spiderweb-like structures" and are included in the general "*corpora lutea*" term and descriptive statistics. The spiderweb-like structures measured between 1,50 and 3,00 cm in largest diameter (table 23), and wall thickness fell between 0,20 and 0,30 cm. These structures were found in the ovary which had zero follicles

Figure 29: Sonogram of a spiderweb-like structure. Scale of 3,0 cm. Copyright IZW.

3 out of 4 times, while the contralateral ovary presented growing follicles. On one occasion, due to previous ultrasound examination performed by another specialist invited by BORA (Dr. Terri Roth on the 22nd of March 2012) it became clear the spiderweb-like structure had increased in size from approximately 2,5 to 3,0 cm in 6 days, while 2 follicles regressed or gave origin to large dense CLs.

Table 23: Quantitative description of obtained data from all females regarding each type of luteal structure, in centimetres and percentage (%).

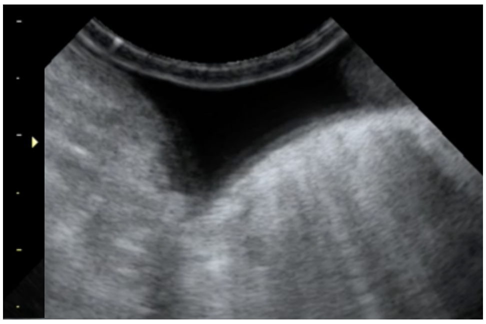
	Dense	Central clot	Spiderweb-like
Mean	1,36	1,42	2,43
SEM	0,10	0,11	0,35
Minimum	0,52	0,60	1,50
Maximum	2,82	1,88	3,00
Sample size	41	12	4
Frequency in Gelugob	100 % (2/2)	-	-
Frequency in Puntung	63,4 % (26/41)	26,8 % (11/41)	9,8 % (4/41)
Frequency in Iman	92,9 % (13/14)	7,1% (1/14)	-

As a last note, multiple hyperechoic spots measuring between approximately 0,20 and 0,50 cm were always visible on both of Puntung's ovaries.

3.8. Other results

In all females under study, large collections of free fluid were observed within the abdominal cavity. These accumulations of peritoneal fluid appeared as angular anechoic areas between and surrounding internal organs such as the uterus and ovaries (fig. 30). In Gelugob this excess was observed at least in 25% of the examinations (4/16); in Puntung it was identified in 44% of examinations (7/16); and in Iman in 67% of examinations (4/6) although fluid collections were less pronounced than in Puntung. Collections of free abdominal fluid were not observed in the males, although the testicles and *cauda epididymidis* were constantly surrounded by an anechoic layer of scrotal fluid (page 68).

Figure 30: Sonogram showing a collection of free fluid in Puntung's abdomen. Scale of 2,5 cm. Copyright IZW.



4. Hormonal profiles of the females and correlation with ultrasonographic findings

Blood progesterone values were obtained on 308 days for Puntung and 35 days for Iman between December 2011 and April 2015. Distribution of hormonal assessments per year can be seen on table 24. Maximum concentration of blood progesterone was 3,04 ng/ml for Puntung and 0,99 ng/ml for Iman. Detection limit of the test was 0,15 ng/ml by which time progesterone levels were considered baseline. Distribution of assessments in regard to progesterone concentration is shown on table 25.

Table 24: Distribution of blood progesterone assessments per year between December 2011 and April 2015.

Year	Total	2011	2012	2013	2014	2015
Puntung	308	1	143	131	32	1
Iman	35	-	-	-	34	1

Table 25: Distribution of blood progesterone assessments (total number and percentage) in regard to progesterone concentrations (in ng/ml).

Progesterone	< 0,15		0,15 > 1,0		1,0 > 2,0		2,0 > 3,0		> 3,0	
Puntung	6	1,9%	186	60,4%	90	29,2%	25	8,1%	1	0,3%
Iman	1	2,9%	34	97,1%	-		-		-	

Blood progesterone values could only be correlated with ultrasonographic examinations performed by the IZW team on 9 examination days of Puntung and 1 of Iman. The quantifications corresponded to the same day as the examination on 6 out of 10 times, while the other 4 values were from the day immediately before or after. While no probable associations were noted when combining progesterone levels with ultrasonographic findings on the reproductive tract, several were evident between blood progesterone concentrations and ovarian functional structures.

4.1. Correlation with ovarian functional structures

Starting with the luteal structures, large dense CLs measuring between 2,30 and 2,82 cm were observed in combination with blood progesterone values ranging from approximately 0,59 to 2,23 ng/ml (n=6). These largest CLs were accompanied by 1 to 3 other CLs measuring between 1,0 and 1,72 cm. Spiderweb-like structures (with 2,26 and 2,25 cm) were observed in combination with 2 to 3 dense CLs measuring between 0,90 and 1,90 cm and blood progesterone values of 1,25 and 1,28 ng/ml.

Blood progesterone values between 0,5 and 1,0 ng/ml were associated with dense CLs of approximately 2,45 cm along with 2 to 3 other CLs measuring 1,0 to 1,5 cm. Blood progesterone values between detection limit and 0,5 ng/ml were associated with 1 medium CL measuring 1,6 to 1,7 cm along with 1 to 3 other dense CLs measuring 0,55 to 0,75 cm.

Moving on to follicular structures, they were observed with progesterone values ranging from detection level to 2,23 ng/ml (n=9). Largest or future dominant follicles measuring between 1,05 and 1,83 cm were observed with blood progesterone values above 1,59 ng/ml. Follicles over 2,0 cm were associated with blood progesterone values below 1,0 ng/ml.

In summary, when considering the largest dense *corpus luteum* and the largest follicle or known selected dominant follicle (two previously stated cases on page 50) in each of the examination days, some patterns could be observed. The 10 sets of data (progesterone value, CL dimension and follicle dimension) appear to separate in three types of combinations (table 26):

1) medium progesterone (≈ 1,20 to 1,30 ng/ml) + medium *corpus luteum* (≈ 1,90 cm) + small follicle (≈ 1,00 cm);

2) high progesterone (≈ 1,60 to 2,20 ng/ml) + large *corpus luteum* (≈ 2,00 to 2,80 cm) + medium follicle (≈ 1,20 to 1,80 cm);

3) low progesterone (below 1,00 ng/ml) + large *corpus luteum* (≈ 1,70 to 2,40 cm) + large follicle (≈ 2,00 to 2,30 cm).

Table 26: Summary of apparent combinations between blood progesterone values, largest dense *corpus luteum* and largest follicle or known selected dominant follicle.

Progesterone value	Largest dense *corpus luteum*	Largest or dominant follicle
Medium	Medium	Small
Large	Large	Medium
Small	Large	Large

5. Reproductive procedures conducted in female rhinoceroses

5.1. Hormonal treatments

Several different hormonal protocols were employed in order to promote follicular growth, follicle maturation, ovulation, estrous synchronization and superovulation. A specific analysis of each protocol and respective results follows.

Table 27: Summary of hormonal treatments applied to Gelugob. Hormonal agents: equine and human chorionic gonadotropin (eCG and hCG), equine and human follicle stimulating hormone (eFSH and hFSH), human luteinizing hormone (LH).

Date	Agents	Protocol	Goal	Results
January 11	eCG	3000 IU	stimulation of follicular growth	x
January 11	hFSH/LH	225 IU/ 24h x 2 days		x
October 11	eFSH hCG	12,5 mg/ 12h x 6 days 1 day of pause 5000 IU		x

During the study period Gelugob underwent 3 hormonal treatments to stimulate follicular growth as described on table 27. Reported effects of eCG administration were increased activity and tension, decreased food intake and enlargement of the vulva and clitoris. Observed ultrasonographic changes included slightly activated uterus and ovaries, with hypoechoic endometrium, hypoechoic ovarian cortex, and enlarged vascular network of the ovaries. However, no follicular development was observed on the 5 days following eCG administration.

As a second try, Gelugob received hFSH/LH which also did not result in follicular growth. No pathological changes were observed in the short or long term following this intervention, except for the slight enlargement of previously detected endometrial cysts. The third and last protocol included eFSH and hCG as described on table 27, and also failed at stimulating follicular development. By late 2011 Gelugob was considered to be in a post-reproductive phase and therefore no longer able to breed.

Table 28: Summary of hormonal treatments applied to Puntung. Hormonal agents: prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), gonadotropin-releasing hormone agonist deslorelin (GnRH), human chorionic gonadotropin (hCG), chlormadinone acetate (CMA), equine follicle stimulating hormone (eFSH), altrenogest. Abbreviations: *corpora lutea* (CLs), artificial insemination (AI).

Date	Agents	Protocol	Goal	Results
February 12	$PGF_{2\alpha}$ GnRH	-	regression of CLs and follicular growth	x
March 13	hCG	15 000 IU	follicle maturation for oocyte collection	x
June 13	hCG	20 000 IU	follicular growth and ovulation for AI	x
May - Jul 14	CMA eFSH	30 mg/ 24h x 49 days 3 days of pause 12,5 mg/ 12h x 2 days	estrous synchronization and superovulation	x
Sept - Oct 14	altrenogest	44 days	estrous synchronization	x

In what concerns Puntung, during the study period she was subjected to 5 hormonal treatments as described on table 28. The first intervention included $PGF_{2\alpha}$ to induce the regression of *corpora lutea* and GnRH agonist deslorelin to stimulate follicular growth (doses unknown to the author). In the 6h following administration the luteal structures started to regress: 2,26 to 1,90 cm spiderweb-like structure; 1,90 to 1,82 cm and 0,96 to 0,93 cm dense CLs. However, when 24h had passed only 2 small follicles measuring 0,38 and 0,25 cm could be seen in the ovaries. Regarding serum progesterone values, on the day of administration it was quantified as 1,28 ng/ml and 3 days later as 1,36 ng/ml. After this attempt it was agreed the team should focus on improving the condition of Puntung's uterus (see ahead page 58).

In March 2013 the IZW team had already understood that natural breeding would be very difficult for Puntung, and an attempt at transrectal follicle aspiration and oocyte collection was performed for the first time in a Sumatran rhinoceros. Upon observation of one large irregularly shaped follicle (2,32 cm in largest diameter) and one medium follicle (1,20 cm), hCG was employed to support final growth and maturation. On the day of collection the largest follicle measured 2,16 cm but presented a regular shape, and the second follicle had reduced to 1,08 cm. Serum progesterone was later quantified as 0,71 ng/ml before hCG administration and 0,59 ng/ml after collection, increasing in the following days.

In June 2013 the plan was to perform AI in Puntung following the successful aspiration of large endometrial cysts. Ultrasonographic examination revealed the presence of 2 medium follicles (1,28 and 1,22 cm) and 1 small follicle (0,56 cm) along with 3 large CLs. Three days later the largest follicle was only 1,83 cm and hCG was employed to stimulate follicular growth and induce ovulation. Artificial insemination took place 20h later, when the largest follicle was still 1,83 cm and the second largest had reduced to 0,84 cm. Serum progesterone was later quantified as 2,23 ng/ml before hCG administration and 1,83 ng/ml on the day of AI.
Seeing how difficult it was to understand the estrous cycle of Puntung and coordinate the visits of the IZW team, in 2014 it was decided to make an attempt at estrous synchronization and superovulation. The outlined protocol using CMA and eFSH is shown on table 28. Length of the treatment was decided as minimum 40 days bearing in mind the apparent length of Puntung's estrous cycle and previous experience of the IZW team with this protocol in other rhinoceros species. Serum progesterone values went down from 2,71 ng/ml to baseline values within 2 weeks, where they stayed with little variation. Unexpectedly, Dr. Zahari Zainuddin reported several follicles growing and regressing throughout the treatment. At the end of the protocol, ultrasonographic examination by the IZW team revealed only 1 medium (1,22 cm) and 3 small follicles (0,31 to 0,50 cm) in Puntung's ovaries, along with 1 to 2 figures resembling CLs. Furthermore, countless endometrial cysts were observed representing a severe decline in Puntung's uterine health.
A second try at estrous synchronization and superovulation took place before the end of the study period, this time with altrenogest (dose unknown to the author, table 28). Follicles were also reported by Dr. Zahari Zainuddin, especially in the first weeks. Serum progesterone never reached baseline, and instead fluctuated between 0,15 and 0,58 ng/ml reaching once 1,20 ng/ml. Due to lack of effectiveness the treatment was suspended. On the following day Dr. Zahari Zainuddin reported only 2 to 3 follicles measuring up to 1,0 cm, in association with 2 luteal structures and serum progesterone levels of 0,43 ng/ml.

Table 29: Summary of hormonal treatments applied to Iman. Hormonal agents: chlormadinone acetate (CMA), equine follicle stimulating hormone (eFSH), altrenogest.

Date	Agents	Protocol	Goal	Results
Jun - Jul 14	CMA eFSH	30 mg/ 24h x 32 days 3 days of pause 12,5 mg/ 12h x 2 days	estrous synchronization and superovulation	x
Sept -Oct 14	altrenogest	44 days	estrous synchronization	x

During the study period Iman underwent 2 hormonal treatments as described on table 29. First, CMA and eFSH were used to induce estrous synchronization and superovulation. Serum progesterone values oscillated between 0,20 and 0,30 ng/ml, never reaching basal levels.

Dr. Zahari Zainuddin reported several follicles growing up and regressing during the treatment. At the end of the protocol, ultrasonographic examination by the IZW team revealed 8 follicles between 0,94 and 2,30 cm along with 2 dense CLs. A second try at estrous synchronization took place with altrenogest. During the treatment period, serum progesterone varied between 0,18 and 0,52 ng/ml, and several follicles were reported by Dr. Zahari Zainuddin. The treatment was suspended due to lack of effect, and on the following day Dr. Zahari Zainuddin reported 4 follicles between 0,50 and 2,50 cm and serum progesterone of 0,46 ng/ml.

5.2. Procedures aiming at the removal of endometrial cysts

To improve Puntung's uterine condition, four different techniques were employed for the removal of endometrial cysts including lavages, cyst photoablation and aspiration (table 30).

Table 30: Summary of techniques employed to remove endometrial cysts in Puntung.

Date	Technique	Goal	Outcome
March 12	uterine flushing with TCM-199	endometrial regeneration	✓
June 12	endoscopic-assisted laser photoablation	rupture of large cysts	x
June 12	uterine flushing with povidone-iodine	non-path inflammation and regeneration	x
March 13	ultrasound guided transrectal aspiration	aspiration of large cysts	✓

For the first procedure, the uterus was flushed with tissue culture medium 199 (TCM-199, Sigma Aldrich GmbH, Munich, Germany; fig. 31 and 32) causing some cysts to rupture and promoting endometrial regeneration. A course of antibiotics was given following the procedure. When Puntung was reassessed 3 months later, ultrasonography revealed a significant improvement in extent and severity of the endometrial cystic hyperplasia, with at least one area of the uterus now free of cysts. The larger cysts in both uterine horns remained, however, unaltered.

Figure 31: Puntung undergoing uterine lavage. In the picture Prof. Dr. Thomas Hildebrandt and Dr. Frank Göritz. Copyright BORA/IZW.

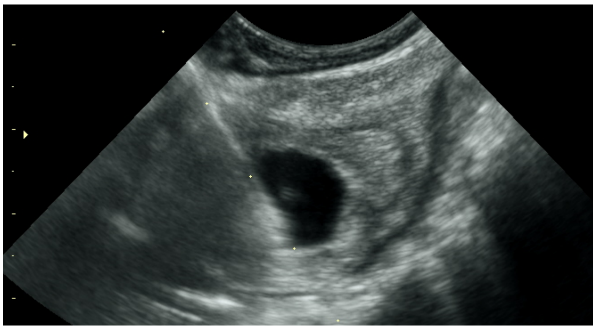
For the following procedure the team decided to rupture the largest endometrial cysts using an endoscope and laser technology, in a procedure termed endoscopic-assisted laser photoablation. When visibility became poor the procedure was assisted with transrectal ultrasonography.

In the same intervention half of the uterus was flushed with povidone-iodine solution (Braunol®, B. Braun Melsungen AG, Germany) promoting non-pathological inflammation and endometrial regeneration, in an attempt to understand which technique bears the best results. A course of systemic antibiotics and oral analgesics was given, but reported reduction in food intake and increase in vocalization may have been signs of discomfort following both procedures. When Puntung was reassessed 5 months later, ultrasonography revealed a significant deterioration in uterine condition with general growth of endometrial cysts.

As a last attempt, transrectal access established for oocyte collection was also used for aspiration of large endometrial cysts (fig. 33). The pathological structures were visibly reduced and obtained fluid was clear, but only 3 months later the large cysts were again evident.

Figure 32: Sonogram of Puntung's uterus during uterine lavage with tissue culture medium 199 showing the endometrial cysts. Scale of 5,0 cm. Copyright IZW.

Figure 33: Sonogram showing puncture and aspiration of a previously represented large cyst in Puntung's uterus (figure 21). Scale of 3,0 cm. Copyright IZW.



5.3. Aspiration of the hydrosalpinx

Transrectal access established for oocyte collection in April 2015 was also used for the aspiration of Iman's hydrosalpinx. Approximately 40 ml of brownish transparent fluid were collected and the pathological structure was visibly reduced. Bacteriological analysis was recommended by the IZW team but results were not returned.

5.4. Artificial insemination

An attempt at artificial insemination in Puntung was planned for June 2013, considering that fertilization and implantation could occur at the cranial ends of the uterine horns following aspiration of the large cysts located there. Close examination of the uterus upon arrival of the IZW team revealed the large cystic formations were once again present, but still the plan went forth. AI took place 20h after hCG administration, when the largest follicle measured 1,83 cm (further information on ovarian structures can be found on page 57). Description of the technique as conducted in Puntung mimicked that previously reported by Hildebrandt *et al.* 2007 (see page 19), using a specially designed catheter (patent DE 10203094A1, Chirurgiemechanik Schnorrenberg, 15569 Woltersdorf, Germany) in association with transrectal ultrasonography. Fresh semen collected from Tam approximately 1h before the procedure was used for deep deposition on the side of expected ovulation. Regarding serum progesterone, it was later quantified as 2,23 ng/ml before hCG administration and 1,83 ng/ml on the day of AI. Fertilization was not achieved as proven by serum progesterone monitoring.

5.5. Oocyte collection

As natural conception seemed less plausible with each reproductive assessment of the females, the focus of the programme shifted to *in vitro* fertilization. The newly developed technique of ultrasound-guided transrectal "ovum pick-up" was initially chosen for the collection of oocytes in Sumatran rhinoceroses due to its unprecedented success in other rhinoceros species. Description of the technique as conducted in Puntung and Iman corresponded to that previously reported by Hermes *et al*. 2009b (see page 19), using a specialized device to hold both the custom-made needle and a micro-convex ultrasound transducer. In 2014 the IZW team invited Prof. Dr. Cesare Galli (Avantea, Italy), a renowned specialist in assisted reproduction and reproductive biotechnologies in domestic animals, to assist with the procedures. Due to the small size of female Sumatran rhinoceroses and extensive experience of Prof. Dr. Galli with this technique, the collections were conducted through transvaginal access. Preparation of the animal was similar but the specialized needle-holder was instead used in the vagina while transrectal palpation of the ovary was conducted (fig. 34 and 35).

In all oocyte collections females were placed under full anesthesia in either lateral or sternal recumbency, favouring the ovary of interest or providing equal access to both ovaries. Follicular fluid was aspirated into embryo flushing medium (Euroflush®, IVM Technologies, France) supplemented with heparin (fig. 36). Postsurgical wound management consisted in rectal or vaginal placement of oxytetracycline foaming tablets (Terramycin Foaming Uterus Tablets, Pfizer, Germany). A summary of all interventions is shown on table 31 and a description of each collection follows below.

Table 31: Summary of oocyte collections in Puntung and Iman. Abbreviations: transrectal access (TR), transvaginal access (TV), *cumulus*-oocyte complexes (COCs).

Date	Animal	Access	Follicles present: number and diameter		Follicles aspirated	COCs recovered
March 13	Puntung	TR	2	2,16 - 1,08 cm	2	0
May 14	Iman	TV	4	1,69 - 0,90 cm	4	3
July 14	Iman	TV	8	2,30 - 0,94 cm	3	2
April 15	Puntung	TR	3	1,99 - 0,56 cm	2	0
	Iman	TR	4	2,02 - 1,06 cm	4	3

In March 2013 took place the first attempt at follicle aspiration and oocyte collection in a Sumatran rhinoceros. Puntung presented 2 follicles whose maturation was supported by hCG. Aspiration was successful but coagulation of the follicular fluid made it impossible to recover the oocytes. On the following attempts the flushing medium was supplemented with heparin.

In May 2014 Puntung presented only 1 small follicle while Iman's ovaries held 4 follicles between 0,90 and 1,69 cm. Follicle aspiration was performed in Iman through transvaginal access, and 3 COCs were identified and recovered.

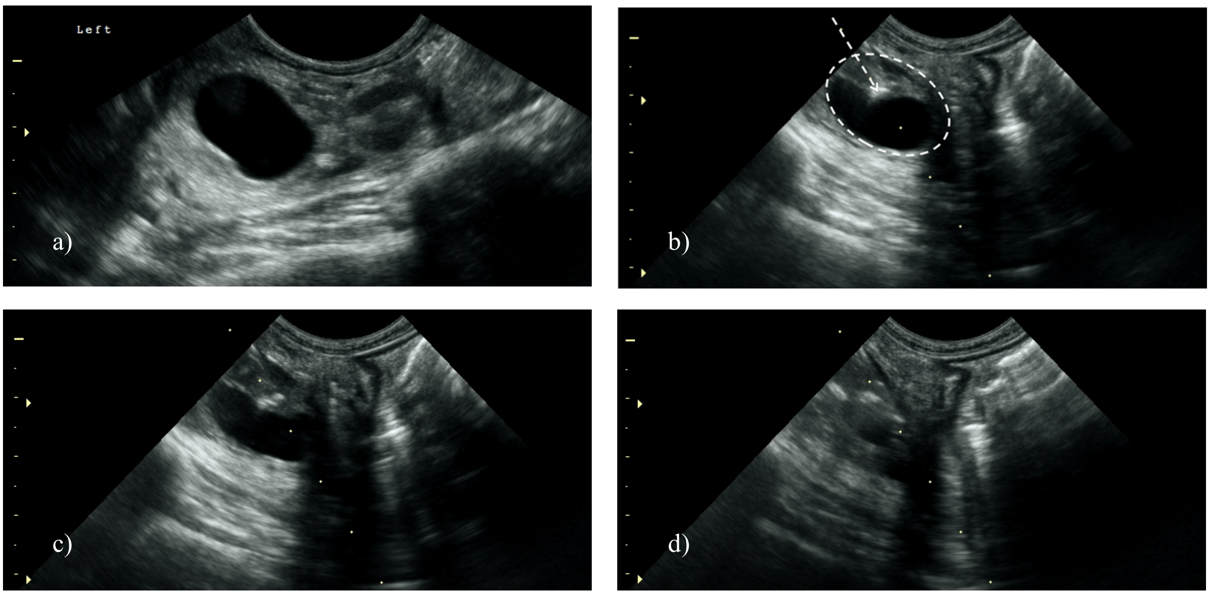
In July 2014 after the first long synchronization and superovulation treatment, Puntung exhibited 4 follicles between 0,31 and 1,22 cm while Iman presented 8 follicles between 0,94 and 2,30 cm. Follicle aspiration was again performed in Iman by transvaginal access. A total of 3 follicles were aspirated and 2 COCs were recovered.

On the last trip of the study period, Puntung presented 3 follicles between 0,56 and 1,99 cm while Iman presented 4 follicles between 1,06 and 2,02 cm (plus one with 0,25 cm). The 2 largest follicles from Puntung were successfully aspirated through transrectal access, but oocytes were impossible to identify and recover. In Iman's case, transrectal collection of all 4 follicles was successful, as was the recovery of 3 COCs.

Figure 34: Oocyte collection in Puntung. In the picture Prof. Dr. Thomas Hildebrandt and Dr. Robert Hermes. Copyright BORA/IZW.

Figure 35: Oocyte collection in Iman. In the picture Prof. Dr. Cesare Galli and Prof. Dr. Thomas Hildebrandt. Copyright BORA/IZW.

Figure 36: Sonograms representing the procedure of follicle aspiration: a) large follicle in Puntung's left ovary; b) follicle and needle trajectory delineated; c) puncture of the follicle; d) aspiration and collapse of the follicle. Scale of 3,0 cm in a) and 4,0 cm in b) c) d). Copyright IZW.



5.6. In vitro maturation and intracytoplasmic sperm injection

After recovery, *cumulus*-oocyte complexes underwent IVM in supplemented maturation medium, and *cumulus* cells were removed (fig. 37). Semen samples from Tam were thawed and prepared, and ICSI was performed in maturated oocytes using one single spermatozoon. The oocytes were then activated and cultured. Specifics of these technologies are beyond the scope of the present study and will not be addressed. A summary of all *in vitro* procedures and respective outcome is shown on table 32, and a description of each intervention follows bellow.

Table 32: Summary of *in vitro* procedures. Abbreviations: *cumulus*-oocyte complexes (COCs), *in vitro* maturation (IVM), *cumulus oophorus* (CO), germinal vesicle breakdown (GVBD), intracytoplasmic sperm injection (ICSI).

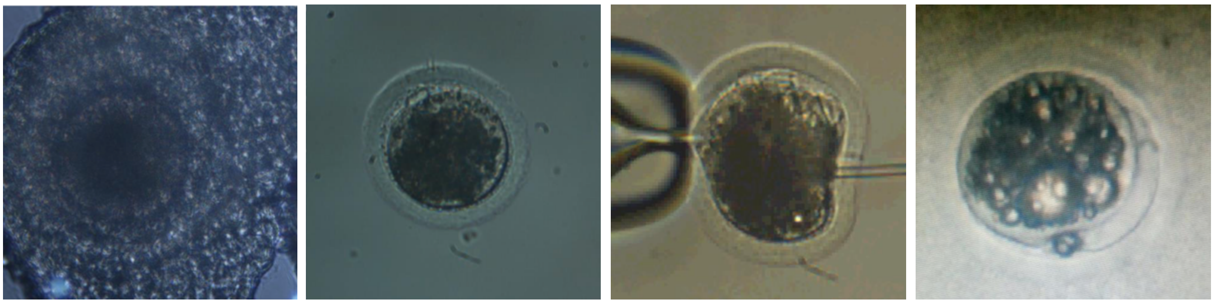
Date	Animal	COCs	IVM results		ICSI	cleavage
May 14	Iman	3	1 compacted CO 2 expanded CO	2 metaphase II	2	0
July 14	Iman	2	1 compacted CO 1 expanded CO	1 GVBD 1 metaphase I	0	0
April 15	Iman	3	-	3 metaphase II	1	0

In May 2014 a total of 3 COCs were recovered from Iman. The cells were held at room temperature for 24h and then underwent IVM at 34ºC for 34h. Further work was conducted by Prof. Dr. Galli and Dr. Giovanna Lazzari at Avantea's laboratory in Italy. After IVM, 2 of the oocytes presented an expanded *cumulus oophorus* while 1 had a compacted *cumulus*. ICSI was performed on the 2 oocytes which achieved metaphase II. Presumptive zygotes were stained with Hoechst dye 72h after ICSI. Both oocytes revealed condensed chromatin but no *pronuclei*, which may be a sign of degeneration. Cleavage was not achieved.

On the following attempt 2 COCs were collected from Iman. The cells were held at room temperature for 5h and underwent IVM at 38,5ºC for 28h. Further work was again conducted at Avantea in Italy. One of the oocytes presented an expanded *cumulus oophorus* while the other remained compact, and both were stained with Hoechst dye. One of the oocytes proved to be in germinal vesicle breakdown stage (GVBD), while the other reached metaphase I but also did not maturate further. Due to poor quality of the oocytes, ICSI was not performed.

On the last trip under study 3 COCs were recovered from Iman. Further work was this time conducted by Prof. Dr. Arief Boediono from Bogor Agricultural University (Bogor, Indonesia) at the Agro-Biotechnology Institute (ABI) in Kuala Lumpur, Malaysia. After IVM and removal of *cumulus* cells all oocytes were determined to be in metaphase II. Frozen-thawed semen samples from Tam were prepared, but spermatozoa were reported to be dead. ICSI was performed only on 1 oocyte with a dead sperm cell, and the other 2 oocytes were vitrified. Two days after ICSI cleavage was not achieved and the oocyte showed signs of fragmentation.

Figure 37: From left to right: *cumulus*-oocyte complex collected from Iman in April 2015; oocyte after IVM and removal of *cumulus* cells; intracytoplasmic sperm injection; fragmented oocyte two days after ICSI. Courtesy of Prof. Dr. Thomas Hildebrandt and Prof. Dr. Arief Boediono. Copyright BORA/IZW.



5.7. Cell culture

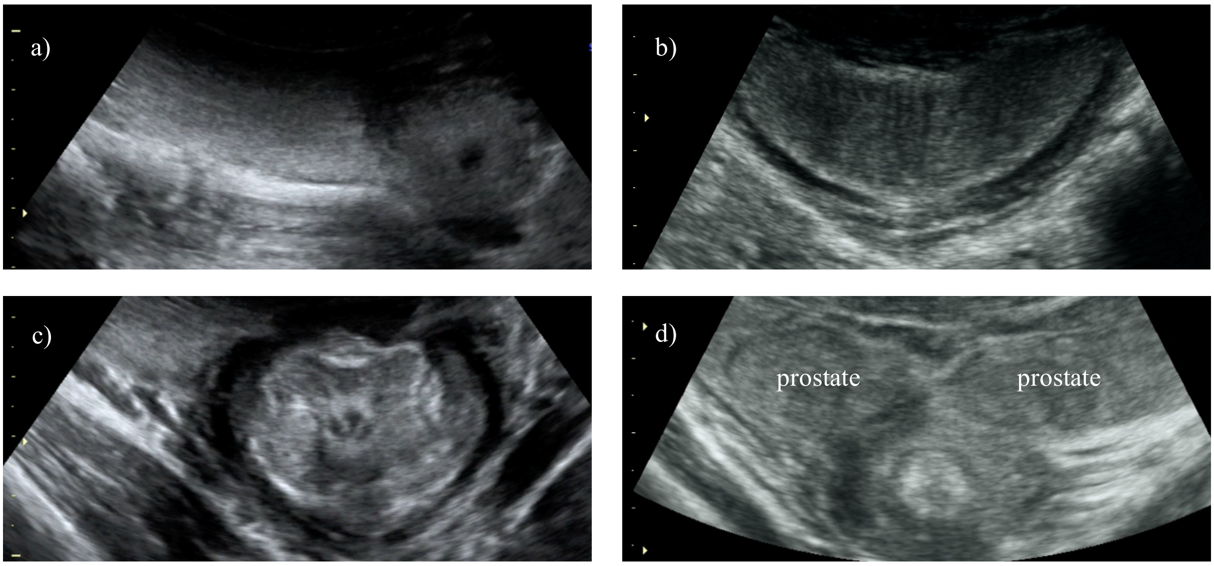
Cell cultures were established from skin and mucosa samples of all Sumatran rhinoceroses kept at the BRS, to preserve their genome and aim at the development of induced pluripotent stem cells (iPSCs). Samples were collected from various points including ear, mouth, neck, axilla and genital area; from Gelugob and Tam in October 2011, and from Puntung, Iman and again Tam in May 2014. Establishment of cell cultures and further work was conducted by renowned specialists in the field, namely Prof. Dr. Katarina Jewgenow (IZW, Berlin), Dr. Vasil Galat (Stanley Manne Children's Research Institute of Northwestern University, Chicago, USA), Dipl. Biol. Matthias Lenk (Friedrich-Loeffler Federal Research Institute for Animal Health, Germany) and Dr. Oliver Ryder (San Diego Zoo's Institute for Conservation Research, USA).

6. Ultrasonographic findings in male rhinoceroses

6.1. Urethra

When performing transrectal ultrasonography in a male rhinoceros, the first structure to be located and examined is the pelvic urethra enclosed in the pelvic canal, followed by the prostatic urethra surrounded by the prostate gland. In short-axis transverse view, the pelvic urethra presents two different ultrasonographic appearances. The most caudal portion of the pelvic urethra is identified as a round structure surrounded by a thin layer of hypoechoic tissue (*urethralis* muscle). This narrow part appears to be short in length and is perceived as the external urethral constriction or sphincter (fig. 38a). Cranially to this portion and before reaching the prostate, the mucosa of the urethra expands into a structure with various mucosal folds, represented by alternating hyper- and hypoechoic vertical lines, also surrounded by hypoechoic tissue (*urethralis* muscle, fig. 38b). Following this part, the prostatic lobes appear and the urethra becomes smaller and round in short-axis transverse section, with a central hyperechoic area surrounded by a thick hypoechoic layer (muscle). This contracted portion is identified as the internal urethral sphincter (fig. 38d). A structure resembling the *colliculus* *seminalis* was observed ventrally to the caudal part of the prostate, and caudally to the internal urethral sphincter. This structure was part of the urethral mucosa and presented 3 openings suggestive of the orifices for the ejaculatory ducts and *utriculus masculinus* (fig. 38c).

Figure 38: Sonograms showing the different portions of the male pelvic urethra in short-axis transverse section. From caudal to cranial: a) external constriction; b) bulbous expansion; c) *colliculus seminalis*; and d) internal constriction or prostatic urethra. Scale of 4,0 cm in a) and c); and 3,0 cm in b) and d). Copyright IZW.



Signs of pathological alterations such as cysts or calcifications were not observed, except for one occasion in 2005 when Tanjung presented small hyperechoic spots (0,2 to 0,5 cm) in the proximity of the prostatic urethra. This finding is suggestive of calcifications occurring in the excretory ducts of the accessory sex glands. Measurements were obtained from Tam's urethra on 7 examinations. At its largest point, the pelvic urethra measured on average 5,87 x 2,97 cm. If only mucosa was measured values dropped to 5,11 x 2,16 cm. When a subtraction was performed between the 2 values in each examination, an estimate measurement of the surrounding muscle layer was obtained, revealing an average thickness of 0,79 cm. Further information on Tam's pelvic urethra can be found on table 33.

Table 33: Quantitative description of obtained data regarding the pelvic urethra of Tam, in centimetres.

	Total		Mucosa		Muscle layer
	Width	Height	Width	Height	
Mean	5,87	2,97	5,11	2,16	0,79
SEM	0,17	0,09	0,13	0,07	0,06
Minimum	5,30	2,74	4,77	1,88	0,59
Maximum	6,45	3,42	5,66	2,41	1,02
Sample size	7	7	7	7	7

The same methodology was applied to Tam's prostatic urethra. At its largest point, average dimensions were 2,60 x 2,29 cm. Values declined to 1,07 x 0,84 cm if only mucosa was taken into account. The surrounding muscle layer was measured indirectly, revealing an average thickness of 1,49 cm. Further information on Tam's prostatic urethra can be found on table 34.

Table 34: Quantitative description of obtained data regarding the prostatic urethra of Tam, in centimetres.

	Total		Mucosa		Muscle layer
	Width	Height	Width	Height	
Mean	2,60	2,29	1,07	0,84	1,49
SEM	0,13	0,10	0,07	0,04	0,08
Minimum	2,16	1,79	0,82	0,67	1,20
Maximum	3,10	2,52	1,32	0,95	1,83
Sample size	7	7	7	7	7

6.2. Urinary bladder

In a cranial progression, the urinary bladder succeeds the previously described internal urethral sphincter. Information regarding the urinary bladder of male rhinoceroses, including measurements and approximate volume of observed deposits of urinary sediment, is reported on page 40 in combination with data from the females.

6.3. Bulbourethral glands

The *glandula bulbourethralis* is a paired accessory gland of the male reproductive system. It can be found caudally on both sides of the pelvic urethra, before the urethral expansion. In the Sumatran rhinoceros these glands appear to be horizontally oblong in shape. Ultrasonographic appearance was moderately echogenic, dense and homogeneous (fig. 39). In 2005, the bulbourethral glands of Tanjung were assessed most often with a linear transducer, apparently in a parasagittal plane (parallel plane to the long-axis vertical cross-section of the pelvic urethra). In this transverse view they were approximately round in shape and measured on average 3,68 cm in height by 4,14 cm in width (table 35, fig. 40). After 2009 Tam was most often assessed with a convex transducer, and his bulbourethral glands were imaged in a transverse plane (short-axis transverse view of the pelvic urethra). Measurements obtained averaged 6,53 cm in length and 1,92 cm in height (table 35, fig. 39). A general growth tendency could be seen in Tam's bulbourethral glands between 4,54 cm in early 2011 and 8,23 cm in late 2012, followed by a reduction in 2013 and 2014. A central vessel of large calibre was observed within the bulbourethral glands of Tanjung, measuring up to 0,40 cm in diameter (fig. 40). In what concerns Tam, central vessels were also visible on some occasions but were never as significant. A hypoechoic layer (muscle) was also possible to observe surrounding the bulbourethral glands of Tanjung. This characteristic was not as patent in Tam, and a hyperechoic line was often visible marking the limit of the glands.

Table 35: Quantitative description of obtained data regarding the bulbourethral glands of Tanjung and Tam, in centimetres, without distinction between left and right sides.

	Tanjung		Tam	
	Width	Height	Height	Length
Mean	4,14	3,68	1,92	6,53
SEM	0,15	0,18	0,09	0,37
Minimum	3,80	3,10	1,46	4,54
Maximum	4,60	4,20	2,33	8,23
Sample size	5	5	10	10

Figure 39: Sonogram of a bulbourethral gland from Tam. Scale of 3,5 cm. Copyright IZW.

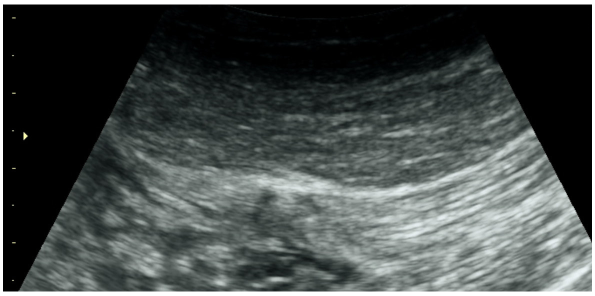
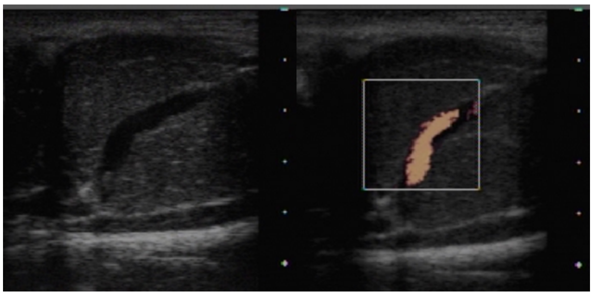


Figure 40: Sonograms of a bulbourethral gland from Tanjung. Right side image showing Doppler mode. Scale of 5,0 cm. Copyright IZW.



6.4. Prostate gland

The prostate or *glandula prostatica* is the male accessory sex gland that appears cranially to the urethral expansion. It is composed of two lobes on each side of the urethra connected by the *isthmus prostatae* surrounding the neck of the bladder. Ultrasonographic appearance was moderately echogenic, heterogeneous with hyperechoic lines in the parenchyma but without anechoic areas. The prostate was sometimes hard to delimit, and other times was surrounded by a hyperechoic line. As previously stated, Tanjung was assessed with linear transducers while Tam was examined with convex probes, resulting in different ultrasonographic views. In Tanjung the prostate was imaged in short-axis transverse view, revealing a triangular shape of the lobes and a shallow elongated isthmus (fig. 42). Obtained measurements from Tanjung in 2005 averaged 3,08 cm in height and 3,57 cm in width (table 36). The isthmus was observed twice in Tanjung measuring 1,30 and 2,00 cm in height. In Tam, obtained images resulted most often from short-axis cross-sections of the urethra, showing elongated prostatic lobes starting in the dorsolateral aspect of the pelvic urethra and surrounding the bladder neck (fig. 41). Measurements obtained from Tam averaged 1,92 cm in height and 5,06 cm in length if both sides are considered together (table 36). Information regarding each prostatic lobe is summarized on table 37. A general growth tendency could be seen in Tam's prostate gland between 3,40 cm in 2009 and 6,30 cm in 2012, followed by a reduction in 2013 and 2014.

Table 36: Quantitative description of obtained data regarding the prostate of Tanjung and Tam, in centimetres, without distinction between left and right lobes.

	Tanjung		Tam	
	Width	Height	Height	Length
Mean	3,57	3,08	1,92	5,06
SEM	0,10	0,09	0,10	0,27
Minimum	3,30	2,80	1,45	3,40
Maximum	3,90	3,40	2,81	6,30
Sample size	6	6	16	12

Table 37: Quantitative description of obtained data regarding Tam's prostate, in centimetres, with distinction between left and right lobes.

	Left		Right	
	Length	Height	Length	Height
Mean	5,23	1,97	4,83	1,87
SEM	0,35	0,15	0,45	0,15
Minimum	4,00	1,50	3,40	1,45
Maximum	6,30	2,81	6,00	2,73
Sample size	7	8	5	8

Figure 41: Sonogram of a prostate lobe from Tam. Note the prostatic urethra in short-axis transverse section on the right side. Scale of 4,5 cm. Copyright IZW.

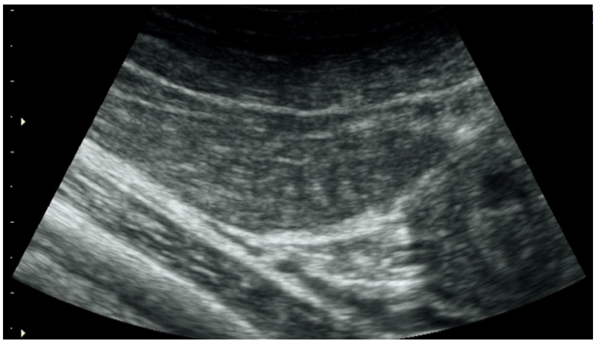
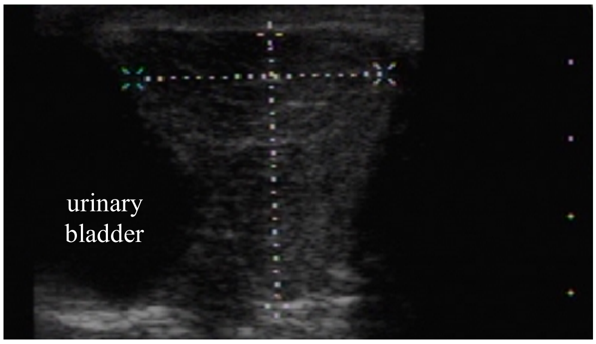


Figure 42: Sonogram of a prostate lobe from Tanjung, in a parallel plane to the long-axis transverse section of the pelvic urethra. Scale of 3,0 cm. Copyright IZW.



6.5. Seminal glands

The *glandula seminalis* is the most cranial paired accessory gland of the male rhinoceros reproductive system. It appears to be elongated and somewhat flattened, and can be found in close proximity to the urinary bladder. Ultrasonographic appearance was most often hypoechogenic (especially in Tam, fig. 43) but sometimes showed hyperechoic streaks increasing the heterogeneity of the parenchyma (especially in Tanjung, fig. 44). Anechoic areas were not observed within the seminal glands. The transversal cross-section of the gland revealed a gross lobulated aspect of its surface. When making no distinction between left and right sides, average length was 5,75 cm for Tanjung and 6,98 cm for Tam, and average height was 1,83 cm for Tanjung and 2,01 cm for Tam (table 38). A third measurement was obtained from Tam twice, with 4,24 and 4,25 cm representing the apparent width of the gland. A general growth tendency could be seen in Tam's seminal glands between 6,26 cm in 2011 and 7,95 cm in 2014, followed by a reduction in 2015.

Table 38: Quantitative description of obtained data regarding the seminal glands of Tanjung and Tam, in centimetres, without distinction between left and right sides.

	Tanjung		Tam	
	Length	Height	Length	Height
Mean	5,75	1,83	6,98	2,01
SEM	0,13	0,14	0,32	0,30
Minimum	5,30	1,40	6,26	1,04
Maximum	6,20	2,30	7,95	2,95
Sample size	6	6	6	7

Figure 43: Sonogram of a seminal gland from Tam. Scale of 5,0 cm. Copyright IZW.

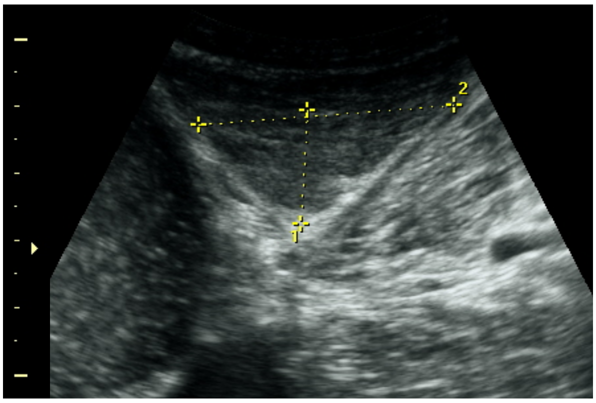
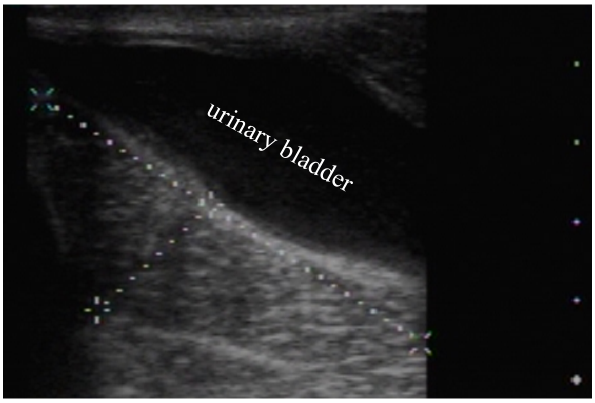


Figure 44: Sonogram of a seminal gland from Tanjung. Scale of 4,0 cm. Copyright IZW.



6.6. Testicles

The *testes* or testicles were assessed with transcutaneous ultrasonography. They are ovoid in shape with an approximately circular short-axis cross-section. Ultrasonographic appearance of the testicles was homogeneous and moderately hypoechogenic, with multiple small (approximately 0,20 cm) hyperechoic *foci* scattered through the lumen (fig. 45). This characteristic was observed in both males, although more intensely in Tam. A hyperechoic line was also observed in the middle of the testicle, corresponding to the *mediastinum testis* and measuring between 0,14 and 0,20 cm in thickness for Tam (average 0,18 cm with n=12, fig. 46). Anechoic areas were often possible to observe surrounding the testicles, in bands of a few millimetres. When these were not visible, a hyperechoic line could be seen delimiting the testicles. No further alterations of structure or echogenicity were noted. Measurements were obtained from Tam on 8 occasions after 2011 with the average values of 3,89 cm for height and 4,14 cm for width of the short-axis cross-section of both testicles without distinction from side (table 39). Length of the testicles was sometimes difficult to assess due to maximum field of view, but with approximate values the average fell on 8,07 cm (table 39). When measurements for each testicle were considered separately, a slight difference could be observed favouring the right testicle (table 40). In regard to Tanjung, measurements were only obtained from the width of the testicles on two occasions in 2005 with 4,20 and 4,30 cm.

Table 39: Quantitative description of obtained data regarding both testicles of Tam, in centimetres.

	Length	Height	Width
Mean	8,07	3,89	4,14
SEM	0,26	0,11	0,10
Minimum	6,70	3,32	3,63
Maximum	10,00	5,30	4,86
Sample size	15	16	14

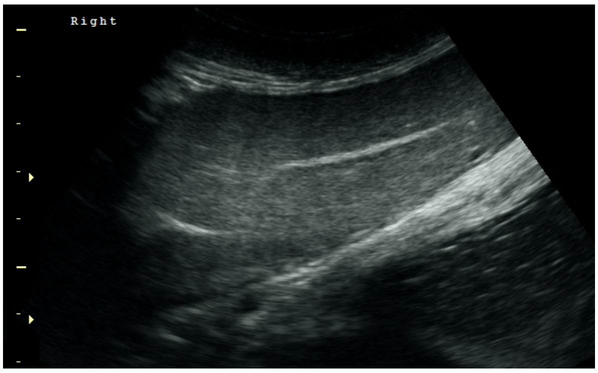
Table 40: Quantitative description of obtained data regarding each testicle of Tam, in centimetres.

	Left			Right		
	Length	Height	Width	Length	Height	Width
Mean	7,91	3,89	4,16	8,26	3,90	4,12
SEM	0,36	0,22	0,15	0,40	0,09	0,13
Minimum	6,80	3,32	3,80	6,70	3,65	3,63
Maximum	9,50	5,30	4,86	10,00	4,30	4,50
Sample size	8	8	7	7	8	7

When all 3 measurements were obtained from each of Tam's testicles, it was possible to calculate the approximate testicular volume as the volume of an ellipsoid* with length, height and width corresponding to the three axes. Average values obtained were 67,7 cm^3 (n=7) for the left testicle and 74,7 cm^3 (n=6) for the right, with general average at 70,9 cm^3 (n=13).

Figure 45: Sonogram of a testicle from Tam. Note the hyperechoic spots. Scale of 8,0 cm. Copyright IZW.

Figure 46: Sonogram of a testicle from Tam. Note the hyperechoic *mediastinum testis*. Scale of 7,0 cm. Copyright IZW.



6.7. Epididymides

The epididymides were most often difficult to identify and delimit due to large anechoic accumulations surrounding them (fig. 48). This presumably pathologic characteristic termed hydrocele testis was visible in both males, with deposits reaching 1,50 cm in width and 3,0 cm in length close to the *cauda epididymidis* (fig. 49). Although the tail was separated from the testicle due to the anechoic accumulations, the attachment of the epididymal body was maintained. Ultrasonographic appearance of the epididymides was relatively homogeneous and hypoechoic in comparison with the testicles (fig. 47). No further alterations of structure or

* volume of an ellipsoid with a, b and c as the major, minor and vertical axes = (1/6)πabc

echogenicity were noted. Epididymal measurements were obtained on 3 different occasions for Tanjung and 8 for Tam, but in a very inconsistent way. Without distinction from side, the *cauda epididymidis* measured on average 1,70 x 2,53 cm for Tanjung and 1,66 x 2,12 cm for Tam (table 41). From Tam's 3D scans it was possible to obtain measurements for the *corpus epididymidis* and for the diameter of the large artery that supplies the epididymis. The epididymal body was observed in short-axis cross-section of the testicle, and measured on average 2,60 cm in width and 0,84 cm in height (table 41). The epididymal artery could be observed within the ecographic structure of the epididymal body and measured between 0,28 and 0,41 cm in diameter, once reaching 0,50 cm (average 0,37 cm with n=6).

Table 41: Quantitative description of obtained data regarding the epididymides of Tam and Tanjung, in centimetres, without distinction between sides.

	Tanjung		Tam			
	Cauda epididymidis		*Cauda epididymidis*		*Corpus epididymidis*	
	Length	Width	Length	Width	Width	Height
Mean	2,53	1,70	2,12	1,66	2,60	0,84
SEM	0,13	0,24	0,11	0,06	0,10	0,05
Minimum	2,20	1,30	1,80	1,40	2,28	0,73
Maximum	2,80	2,30	2,50	1,81	2,94	1,05
Sample size	4	4	6	6	6	6

Figure 47: Sonogram of a *cauda epididymidis* from Tanjung. Scale of 4,0 cm. Copyright IZW.

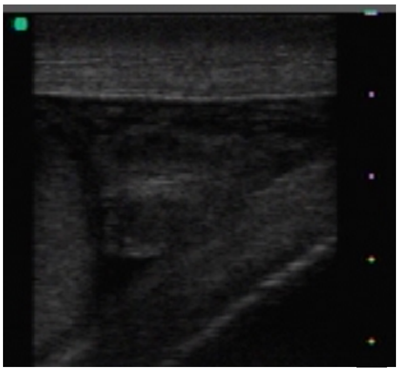


Figure 48: Sonogram of a *cauda epididymidis* from Tanjung, next to a large accumulation of fluid. Scale of 5,0 cm. Copyright IZW.

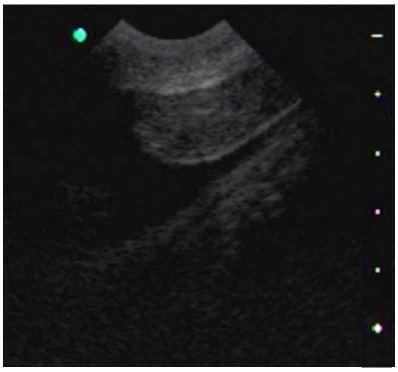
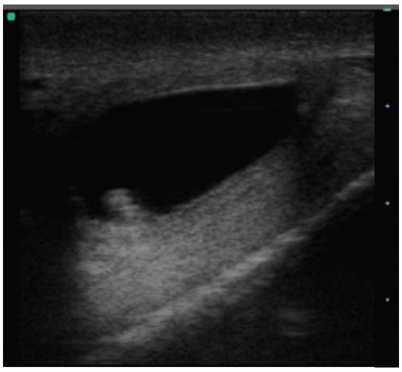


Figure 49: Sonogram of a large accumulation of fluid next to the *cauda epididymidis* of Tanjung. Scale of 3,0 cm. Copyright IZW.



7. Reproductive procedures conducted in male rhinoceroses

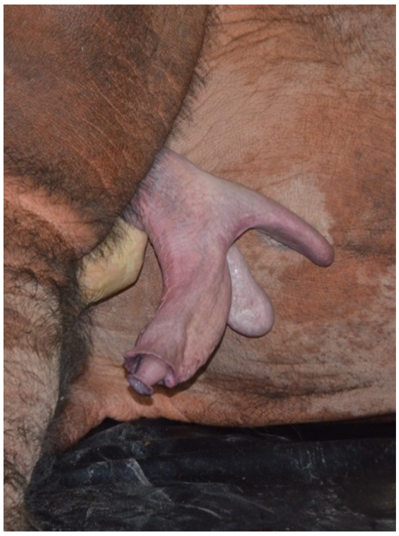
7.1. Semen collection

Semen was collected from Tam by the IZW team a total of 8 times on different occasions between 2009 and 2015. Description of the procedure as conducted in Tam mimicked that previously reported by Hermes *et al.* 2005 (see page 28, fig. 50 and 51), using a combination of electrical stimulation and manual massaging of the accessory sex glands, pelvic and penile urethra. Electroejaculation was performed using a Seager model 14 electroejaculator (Dalzell

USA, Medical Systems, The Plains, VA, USA) and a custom-made rhinoceros electrical probe. Usually a total of 4 to 5 sets of 4 to 5 electrical stimuli were performed with increasing voltage (3-15 V) and amperage. Ejaculation occurred between electrical stimulations, when massaging was being performed. Collected semen fractions were kept at body temperature until processing. Information regarding semen collections from Tanjung was not obtained.

Figure 50: Semen collection by electroejaculation in Tam. In the picture Prof. Dr. Thomas Hildebrandt, Dr. Zahari Zainuddin and Dr. Robert Hermes. Copyright BORA/IZW.

Figure 51: Detail of the *glands penis* of a Sumatran rhinoceros (Tam). Copyright BORA.



7.2. Semen evaluation

Once at the improvised laboratory, semen samples were diluted 1:1 with pre-warmed to 37ºC Berliner Cryomedium (BC) supplemented with 6,25% (v/v) DMSO. Semen assessment included total volume, sperm concentration, sperm motility and sperm morphology. Sperm concentration was estimated using an improved Neubauer hemocytometer. Progressive motility of spermatozoa was evaluated after 1:20 dilution of an aliquot (10 µl) in TCM-199, using a phase contrast microscope equipped with a warm stage at 37ºC (Olympus CH 40, Olympus, Hamburg, Germany). Since concentrations were often very poor, motility was assessed in less than the normally required 200 cells. Smears were prepared for the assessment of sperm morphology and acrosome integrity using eosin-nigrosin. General results from the evaluation of Tam's semen samples are shown on table 42 (n=7). On one occasion in 2011, however, Tam yielded less than 0,1 x10^6 spermatozoa in a 22 ml ejaculate.

Table 42: Summary of results from the evaluation of Tam's semen samples (n=7) showing volume, sperm concentration, total sperm count, sperm motility, normal sperm morphology and acrosome integrity. Abbreviations: spermatozoa (spz).

Volume	Concentration	Total sperm count	Motility	Morphology	Integrity
2 - 48 ml	0,4 - 14,0 x10^6 spz/ml	10,0 - 112,0 x10^6 spz/ejaculate	10 - 60 %	10 - 36 %	10 - 64 %

When analysing the timeline, interesting information comes to light. In 2009 Tam produced 48 ml of semen with a concentration of 1,5 x10^6 spz/ml and 55% motility. In the beginning of 2011 however, Tam yielded less than 0,1 x10^6 spz in a total 22 ml ejaculate. This results were associated with a reported decrease in testicular dimensions and consistency (measurements not obtained for the present study). By February 2011 Tam was considered reproductively inactive, most likely due to the absence of reproductively active and fertile rhinoceroses in the area (Gelugob was the only other rhinoceros at the BRS in 2011) and/or stress caused by the constant presence of another rhinoceros when the species is essentially solitary. Since there was no evidence supporting other hypotheses such as seasonality, concomitant health problems or other sources of stress, it was decided to separate Tam and Gelugob at least 100 metres and continue the search for female rhinoceroses in Sabah. Gelugob was moved to an isolated enclosure in September 2011 and by October 2011 a slight improvement was seen in sperm concentration, although motility was still extremely low. A significative improvement was noted in late February 2012, approximately 2 moths after Puntung arrived at the BRS, when Tam produced 10 ml of semen with 1,0 x10^6 spz/ml and 55% motility. Other possible causes for this improvement besides the influence of Puntung were the introduction of regular semen collections by penile massage in the preceding year (performed by Dr. Zahari Zainuddin and not included in the present study), and a collection made by manual massage on the day previous to electroejaculation. Expectations were high but semen parameters were maintained until July 2014 when a higher sperm concentration was obtained (14,0 x10^6 spz/ml in an 8 ml ejaculate), and April 2015 when a larger volume was obtained (45 ml with 1,0 x10^6 spz/ml).

7.3. Semen preservation

Although Tam's semen was largely unsuitable for efficient cryopreservation, which normally requires over 75% motility, it was nevertheless attempted bearing in mind the absence of more fertile semen donors. Chilling of the extended semen was performed slowly until 4ºC prior to freezing. Unexpectedly, motility suffered a quick drop after chilling to between 0% and 10%. This fact made it clear that after cryopreservation semen parameters would be inadequate for artificial insemination, sufficing only for *in vitro* techniques as a few motile or only alive spermatozoa are necessary. The extended semen was frozen in 0,5 ml straws (Minitube GmbH, Tiefenbach, Germany) 2,0 cm over liquid nitrogen vapour (-80ºC) for 15 minutes, before being submerged. Post-thawing parameters were not assessed since very few semen straws were obtained from Tam. Unexpectedly in 2013 the cryopreservation tank where all samples were stored (kept at LKWP, Malaysia) was found to have become void of liquid nitrogen. All semen samples collected since 2009 were therefore tragically lost. In May 2014, when the first ICSI was attempted, post-thaw motility was reported to be under 5%. A small number of straws were obtained and cryopreserved during this visit but again due to lack of communication from the Asian scientists, all samples were lost in early 2015.

7.4. Cell culture

Cell cultures were established from skin and mucosa samples of all Sumatran rhinoceroses kept at the BRS, to preserve their genome and aim at the development of iPSCs. Further information regarding male rhinoceros Tam is reported on page 63 in combination with the females.

DISCUSSION

1. Relevance

The number of individuals included in the present study is undoubtedly small for the extraction of statistically significant conclusions. Nevertheless, the author and fellow researchers believe it is still very relevant given the extreme conservation status of the species, the continuous failure of conservation programmes and the lack of knowledge regarding reproductive physiology. Furthermore, the sample seems to be representative of the captive population since it accounts for half the reproductively active females and a third of the mature males.

2. Method

In regard to the analysis of ultrasonographic examinations, it is expectable that certain points were under or over-estimated by the inexperienced investigator in spite of great effort to increase accuracy and reduce variability. Examples of such difficulties were the identification and localization of anatomic structures in isolated sonograms, and the image artefacts commonly seen in the reproductive tract due to pockets of bowel gas and fluid-filled structures.

3. Ultrasonographic findings in female rhinoceroses

3.1 Structure of the reproductive organs

Collapsed vaginal thickness was not reported in previous literature and the author hypothesizes there may be valuable information behind this parameter, as suggested by the observation of Iman's vagina. Examination of the cervix revealed a long ovoid shape which was not previously mentioned in the literature. Average values for the cervix were generally larger than those reported for the species (Schaffer *et al.*, 1994; Zahari, 1995). In what concerns the uterus, average values for the body were in agreement with the literature (Zahari, 1995) except for Iman which presented a larger diameter. Average values for the uterine horns were generally smaller than previously reported (Schaffer *et al.*, 1994; Zahari, 1995). Regarding the ovaries, Gelugob's were smaller than previous reports for the species (Schaffer *et al.*, 1994; Zahari, 1995; Roth *et al.*, 2001). Average values for the ovaries of Puntung and Iman were within reference values, but maximum observed length was found to be over the maximum reference.

3.2. Function of the reproductive organs

Gelugob presented an inactive endometrium which suffered a notable decrease in percentage of total thickness of the uterus between 52,7% in 2005 and 40,0% in 2012. Gelugob's ovaries were void of functional structures after 2005, and presented dilated blood vessels in the stroma and small fibrotic spots. Hormonal treatments did not succeed in stimulating follicular development and in late 2011, at the approximate age of 30, Gelugob was declared to be in a post-reproductive stage.

On the rare occasions normal uterine structure was possible to observe in the two younger females, endometrium accounted for 50% to 70% of uterine thickness. Further conclusions on uterine status were not possible to obtain due to pathological alterations and lack of data regarding hormonal profiles and estrous cyclicity.

In regard to the ovaries, Iman appeared to have more intense ovarian activity than Puntung since she presented twice the number of follicles. While Puntung showed a clear inverse relationship between number and dimension of follicles, the distribution of Iman's follicles followed a bell-shaped curve. This finding was most likely due to the small number of examinations, and should come closer to that seen in Puntung if the sample was higher. Considering preovulatory dimensions over 2,0 cm it is interesting to see that in Puntung only 2 follicles out of 14 examinations were observed in this last stage. Follicle shape was dependent of adjacent structures as previously mentioned in the literature (Zahari, 1995) and although ovulation was not evident in any of the examinations, systematic changes in shape were not observed at dominant or preovulatory size like reported for both African species (Radcliffe *et al.*, 1997; Radcliffe *et al.*, 2001; Hildebrandt *et al.*, 2007). Finally, growth rates of 0,20 and 0,25 cm/day were calculated for the selected dominant follicles of two natural cycles, which is in agreement with previously published growth rates in White and Black rhinoceroses (Radcliffe *et al.*, 2001; Hildebrandt *et al.*, 2007).

Selection of the dominant follicle

According to the dimensions at which the selected dominant follicle continued to grow at an apparently constant rate, and the largest subordinate follicle started growing at a reduced rate or regressing, the author puts forward the hypothesis that selection of the dominant follicle in the Sumatran rhinoceros occurs between or around 1,00 to 1,20 cm in diameter. Furthermore, the author hypothesizes that several factors influence selection since the largest follicle at one time does not always become the dominant follicle. In 2016 Ginther, Siddiqui and Baldrighi proposed that functional angiocoupling occurs when a follicle and a CL are adjacent to each other, meaning that if either structure signals for an increase or decrease in blood flow, the adjacent structure will be similarly affected. Ginther and colleagues (2016) based their theory on the following conclusions in heifers: 1) during luteal phase the dimensions and colour Doppler signals for blood flow of both the CL and future dominant follicle are greater when they are in the same ovary and adjacent, than when separated; and 2) during luteolysis the largest or future dominant follicle suffers a decrease in dimension and a loss of future dominant status when adjacent to the regressing CL. The author of the present study therefore proposes that a similar mechanism may occur in the Sumatran rhinoceros, since on both described occasions the largest follicles which later regressed giving way to another dominant follicle, were located in the same ovary and adjacent to the largest regressing CL. In regard to the positive influence of a growing CL on a growing future dominant follicle there was not enough data to infer on this point.

Number of corpora lutea

When comparing number of follicles and number of CLs on both ovaries at each examination day, often the number of CLs was higher. Several hypotheses may be behind this finding.
First, *corpora lutea* of the Sumatran rhinoceros may have a structural lifespan longer than one or even more estrous cycles. The most notorious case of physiologically persistent CLs in wildlife occurs in the monoestrous lynx species, namely the Eurasian and Iberian lynxes. According to Painer *et al.* (2014) in these species one CL persists for at least 2 years while a new ovulation gives origin to a new CL. Both CLs produce progesterone throughout the year, suffering only transitory functional luteolysis at the time of estrus and ovulation. Several hypoechoic CLs with various sizes can be seen in the ovaries at one time, in a number directly correlated with age (Painer *et al.*, 2014). On the other hand, pathologically persistent CLs are common in the mare most often due to diestrus ovulations, early embryonic loss and severe damage to the endometrium (extensive loss of surface epithelium, endometrial fibrosis or gland atrophy) such as caused by pyometra (Santos, Bettencourt & Ginther, 2015). While in the lynx folliculogenesis is halted by the constant secretion of progesterone (Painer *et al.*, 2014), in the mare follicular waves still develop but the dominant follicle finally suffers atresia instead of ovulation (although "complications" such as ovulation and HAF formation are noted in some cases; Santos *et al.*, 2015). Progesterone production goes through transient depressions at the end of each follicular wave in about 50% of the cases in mares, and through a long gradual decrease before luteolysis and ovulation finally occur. Functional and structural regression of the persistent CL in the mare occur at the same rate (Santos *et al.*, 2015). Like both these examples Puntung presented quantifiable blood progesterone during most of the time (98% of samples with 38% above 1 ng/ml; if sampling was in fact systematic and not associated with clinical or ultrasonographic findings). Unlike the lynx and similarly to the mare with persistent CLs, Puntung presents follicular development while large CLs are secreting progesterone. Then, like the lynx and unlike the mare, she seems to undergo functional luteolysis (and not only transient depressions) as suggested by the observation of follicles over 2,0 cm in association with large CLs but low to baseline progesterone. Rare observation of preovulatory

sized follicles in Puntung might mean that follicular waves do not reach ovulation like in the mare, but the simultaneous presence of several CLs with different dimensions seems to support the idea that follicles from the females under study do, in fact, undergo ovulation. A final example that can relate to this first hypothesis is the giraffe estrous cycle. According to Lueders *et al.* (2009) in giraffes a fresh CL is seen 1 day after ovulation but progesterone only starts to increase when it reaches 2,5 cm. Maximum progesterone and maximum diameter of 3,5 cm are seen around day 11 and then progesterone starts declining but the CL stays unchanged for 2 to 3 more days. Progesterone reaches baseline 1 to 2 days before ovulation, but the previous CL only disappears in 4 to 5 days after ovulation. In conclusion, function and structure of giraffe's CLs do not follow a direct relationship, and dominant follicles grow under the influence of high concentrations of progesterone (Lueders *et al.*, 2009). In Puntung, like the giraffe, large follicles are associated with high blood progesterone but preovulatory follicles exist in combination with low to baseline progesterone and large CLs. This also means that, like suspected in Puntung, it is possible for follicles to undergo their final growth under the influence of large concentrations of progesterone. Although structural lifespan of CLs in giraffes is much longer than functional duration, they still don't last longer than one or two cycles like it seems to happen in Puntung and is seen in the two previous examples.

As a second hypothesis to justify a number of CLs larger than the number of follicles in the Sumatran rhinoceros, more than one follicle could give origin to CLs in each estrous cycle. In several mammal species attretic follicles can suffer luteinization giving origin to accessory CLs with the same structure and duration as ovulatory CLs. This usually happens when fertilization is successful to help support the pregnancy (Mossman & Duke, 1973). In the elephant however, every cycle gives origin to several accessory CLs. According to Hermes *et al.* (2000b) and Hildebrandt (2006), the first follicular wave and first LH peak in elephants do not result in ovulation. These follicles suffer atresia and later during the second follicular wave (with only one follicle) develop into several CLs. Progesterone starts increasing 2 to 3 days before the second, ovulatory, LH surge. Both ovulatory and accessory CLs regress before the first LH peak of the following cycle, and reduction in progesterone matches structural changes. A small percentage of CLs resulting from luteinization of follicles develop a fluid-filled central cavity. No follicular growth occurs during the luteal phase (Hermes *et al.*, 2000b; Hildebrandt, 2006). Several points of the elephant strategy do not match the findings in Puntung such as lack of follicular development during luteal phase and the association between luteal structure and activity, but this example shows it is possible that follicles which do not ovulate give origin to CLs even without conception.

Bearing all this information in mind, it is possible that findings in Puntung are due to a combination of both hypotheses, and that Sumatran rhinoceros females exhibit similar strategies to those described in the mentioned species. Since CL activity is reported to be strongly correlated with its vascularity (Miro, 2012) it is recommended that colour Doppler studies are employed to further elucidate this point.

In regard to Iman, she presented good ovarian activity with several follicles and also several CLs at one time. Interestingly, Iman's CLs were smaller than Puntung's and progesterone was detectable in 97% of collections but never above 1 ng/ml. One hypothesis is that accumulation of intraluminal fluid in Iman's uterus may promote the secretion of $PGF_{2\alpha}$ from the endometrium, resulting in early or partial luteolysis.

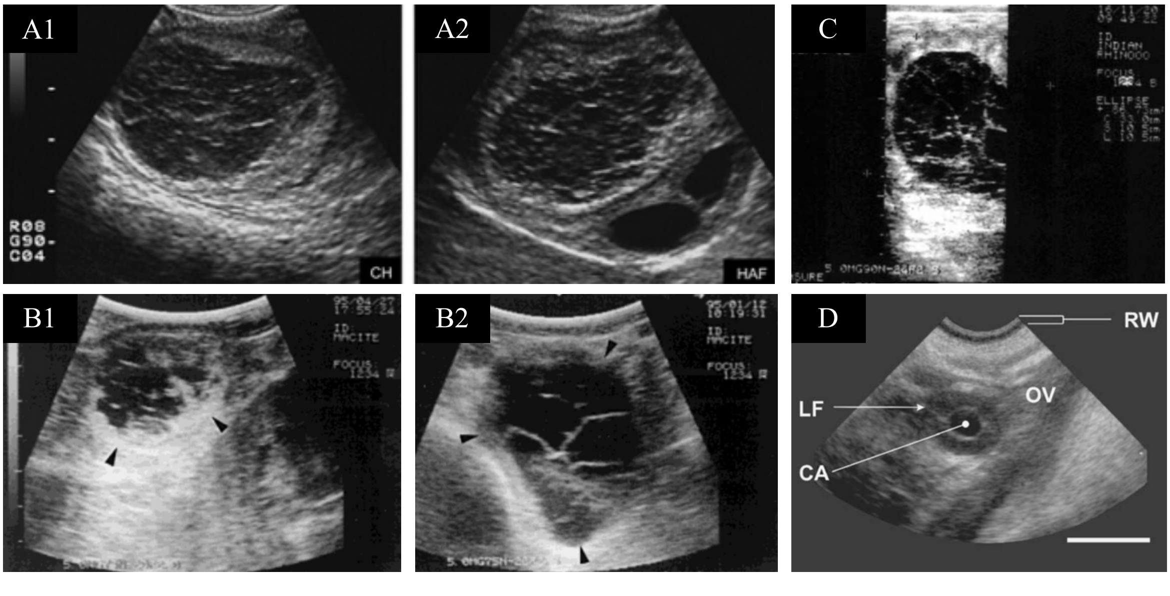
Morphology of corpora lutea

Regarding morphology, three types of luteal structures are described. The most common form was the dense CL, and the description of a fibrous centre is in agreement with reports on other species such as the mare and the elephant (Hildebrandt, 2006; Miro, 2012). On two occasions it was possible to calculate the structural regression rate of dense CLs adjacent to follicles which lost their dominant status. This rate corresponded to 0,50 cm/day when the newly selected dominant follicle was growing on the contralateral ovary, and 0,14 cm/day when it was growing on the ipsilateral ovary (but farther away than the adjacent regressing follicle).

The second most common form was the central clot CL. In mares, approximately 50% of CLs develop a central blood clot which suffers a reduction in non-echogenic area through time. The same mare can produce both types of luteal glands with no difference in concentrations of circulating progesterone (Miro, 2012). These luteal glands with a central blood clot are often termed *corpus hemorrhagicum*, and have been described in the White and Indian rhinoceroses in the same line as the mare (Radcliffe *et al.*, 1997; Stoops *et al.*, 2004; Miro, 2012). In the present study however, reduction in size of the central clot was not observed, and the percentage of hypoechogenic area was approximately the same in every observation. Bearing this in mind it seems the observed central clot CLs are morphologically more similar to the less common elephant CLs which, as described by Hildebrandt (2006), hold a fluid-filled centre and result from the luteinization of follicles (fig. 54D).

The last luteal structure described was the "spiderweb-like structure", which presented thick presumably luteinized walls and a lumen with hypoechoic fluid and fibrinous bands, falling under the definition of *corpora hemorrhagica* for some authors (Radcliffe *et al.*, 1997; Stoops *et al.*, 2004; Ginther *et al.*, 2007) and of hemorrhagic anovulatory follicles for others (Roth *et al.*, 2001; Hermes et al., 2006a; Radcliffe *et al.*, 1997; Ginther *et al.*, 2007). Presumably, the determining factor between the development of a CH or a HAF (since both produce progesterone in a similar way) is the hemorrhagic event occurring after or before the follicle has been emptied of follicular fluid. While in a collapsed follicle blood coagulates and is slowly replaced by luteal tissue, the presence of anticoagulants in follicular fluid prevents its organization when the hemorrhage precedes ovulation (Ginther *et al.*, 2007). Spiderweb-like structures in Puntung appeared to grow over preovulatory size and persist while a new cycle started, as suggested by the observation of one growing over 6 days while other ovarian structures developed and transformed. Bearing in mind all described characteristics and previously mentioned literature, the author believes these are in fact anovulatory hemorrhagic follicles. Formation of HAFs has been described in all rhinoceros species and is frequently associated with non-physiological anestrus in young females (Radcliffe *et al.*, 1997; Radcliffe *et al.*, 2001; Hermes *et al.*, 2006a). In mares, over 85% of HAFs are reported to exhibit thick luteinized walls (Miro, 2012) and in rhinoceroses the presence of HAFs is often paired with progesterone profiles similar to those of ovulatory cycles (Roth *et al.*, 2001; Stoops *et al.*, 2004; Radcliffe *et al.*, 2001). Although Puntung's spiderweb-like structures were always observed in combination with one or more dense CLs (making it difficult to understand if they secrete progesterone), the thickened walls and similarity to previous reports lead to the supposition of these being luteinized HAFs. In conclusion and according to the literature (Radcliffe *et al.*, 1997; Ginther *et al.*, 2007), it is difficult to draw a line between luteinized hemorrhagic anovulatory follicles, *corpora hemorrhagica,* and *corpora lutea* with central clots (fig. 52). Once again, serial ultrasonography and colour Doppler studies of ovarian structures are recommended to further elucidate this matter.

Figure 52: Sonograms retrieved from the referenced literature to exemplify the difficulty in distinguishing the various luteinized structures. A1) equine CH and A2) equine HAF, retrieved from Ginther *et al.* 2007. B1) rhinoceros CH and B2) rhinoceros HAF, retrieved from Radcliffe *et al.*, 1997. C) rhinoceros CH, retrieved from Stoops *et al.*, 2004. D) elephant accessory CL or luteinized follicle, retrieved from Hildebrandt, 2006.



Besides the mentioned luteal structures, Puntung also presented small hyperechoic spots which are compatible with the description of *corpora albicantia* as reported in White and Black rhinoceroses (Schaffer *et al.*, 2001a).

<u>*Induced ovulation*</u>

The final topic for discussion in this chapter is the description of induced ovulation by Roth and colleagues in 2001 (see page 12), which until this day is regarded by the public and part of the scientific community as the most important breakthrough in Sumatran rhinoceros reproduction. Induced ovulation occurs in a variety of mammals (e.g. all camelids, several species of carnivores including most felids, several lagomorphs and some species of rodents) presumably as an evolutionary strategy to prevent wastage of gametes and energy, and enhance results of fertilization, when either the reproduction is seasonal and/or the species is solitary. This way the short window of opportunity is more efficiently used, although in detriment of other evolutionary strategies such as sperm competition (Bakker & Baum, 2000). Induced ovulators present an atypical estrous cycle with only follicular phase while mating does not take place, which results in the absence of luteal glands and quantifiable progesterone production. After mating and ovulation, progesterone levels inhibit estrous cyclicity until pregnancy is established or fails after a period of pseudopregnancy (Bakker & Baum, 2000). In agreement with the general understanding of induced ovulation, main arguments offered by Roth *et al.* (2001) were that ovulation occurred when mating took place and failed when estrus was predicted but the animals were not paired; and that CLs were consequently not observed during periods of absent mating. Roth *et al.* (2001) also reported that anovulatory follicles gave origin to luteinized HAFs with progesterone values similar to normal cycles. Starting with a general analysis of that study, the description of a reproductive strategy unknown to the entire order Perissodactyla without the previous consideration of more likely hypotheses seems like a hasty generalization. Although conception and delivery of the first Sumatran rhinoceros calf in captivity seemed to support the hypothesis at hand, sampling was undoubtedly short especially with only 3 poorly documented failed ovulations and 1 failed LH peak as the exceptions that proved the rule. In regard to observed ovulations it is important to note that at least twice intromission was not achieved, which would be essential even if the stimulus was only seminal fluid. Failed ovulations were poorly documented and could have been related to other factors such as the mentioned storm. In what concerns HAF formation, it has been described in all rhinoceros species and is regarded as non-physiological in all but the Sumatran rhinoceros. Curiously, Roth and colleagues (2001) mention that outside the study period HAFs also occurred when mating took place. Cycle duration with and without ovulation was not reported to be different like it should in induced ovulators, and progesterone did not seem to prevent follicular growth. In regard to the present study, one inconsistency that is immediately clear is the constant presence of CLs in the ovaries of Puntung and Iman without mating during the study period. Furthermore, the present study also reports follicular development while luteal glands are secreting progesterone. It is therefore the understanding of the author that Sumatran rhinoceros do not exhibit induced ovulation. Most likely the unprecedented results obtained by Roth and colleagues were due to serial examinations and introduction of the animals at the right time to allow for successful mating (estrus), besides the necessary health and compatibility between the pair. Nevertheless, the contribution of this team to the captive Sumatran rhinoceros population should be acknowledged and celebrated.

3.3. Pathology of the reproductive organs

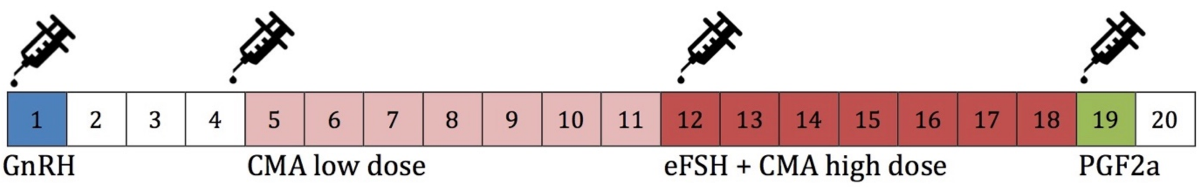
In regard to reproductive pathology, upon capture from the wild and throughout the study period both Puntung and Iman presented clinical and pathological patterns commonly observed in captive female rhinoceroses that have been unable to breed for long periods of time (Hermes *et al.*, 2004; Hermes *et al.*, 2006a; Hermes *et al.*, 2014). Puntung's uterus was severely affected by endometrial cystic hyperplasia with likely hundreds of endometrial cysts reaching 1,60 cm in diameter. Number and dimension of cysts increased through time with only short halts when treatments were applied, revealing a possible positive correlation between pathological changes and age. Cystic hyperplasia of the endometrium like seen in Puntung is often reported in advanced cases in the African species (Hermes *et al.*, 2006a). Endometrial fibrosis and other complications such as hydromucometra also found in advanced cases were not observed in Puntung, which may be related to her young age. Iman in her turn was afflicted by numerous soft tissue masses in the uterus reaching 17,4 cm in diameter, which are consistent with the leiomyomas commonly seen in the cervix and vagina of the Indian rhinoceros and the uterus of African species (Hermes & Hildebrandt, 2012). Iman's tumours showed signs of structured blood supply and areas of necrosis, which is in agreement with clinical reports of regular vaginal discharge of blood, mucus and dead tissue. According to the literature and also with Iman's case, affected animals exhibit only discreet signs such as conception failure and aggressive behaviour until a very advanced stage when vaginal discharge can be seen (Hermes *et al.*, 2016). Interestingly, the vagina and cervix of both females were largely unaffected which also brings the Sumatran rhinoceros closer to the pattern described for both African species (Hermes & Hildebrandt, 2012). Both these conditions are included in a pathological complex described over a decade ago under the term "asymmetric reproductive ageing". It is believed to be a consequence of long non-reproductive periods when continuous ovarian cycle activity leads to prolonged exposure of the reproductive tract to sex steroid hormones, and the development of reproductive pathology (Hermes *et al.*, 2004). In what concerns the Sumatran rhinoceros, going back in time through the literature it is possible to find several reports of reproductive pathology in wild-caught individuals and even wild animals victims of poaching (Schaffer *et al.*, 2001b; Ahmad *et al.*, 2013). The pathological alterations are reported to start at age 10 and become prevalent at age 15, much like the females under study (Schaffer *et al.*, 2001b). An association between these findings and lack of breeding has however never been formally established. Here, the hypothesis under study is that very low densities of a slow breeding solitary species like the Sumatran rhinoceros drastically decrease the possibility of finding a reproductively active partner in the short frame of sexual receptivity, thus leading to long non-reproductive periods in wild individuals.

In what concerns Gelugob, her uterus was mostly free of pathological changes and her ovaries were inactive throughout the study period. Gelugob did not present clear signs of previous parturition, never bred while in captivity since 1994, and was reported to cycle irregularly even before 2005. The last stage of the asymmetric reproductive ageing process is follicular depletion and premature senescence due to continuous follicular output. Non-reproducing middle-aged females like Gelugob are therefore frequently affected by irregular follicular development and erratic luteal activity which worsen with time (Hermes *et al.*, 2004; Hermes *et al.*, 2006a). Hypotheses are that Gelugob developed reproductive pathology early in her life while cycling, which then regressed as the ovaries became less active; that she was breeding before being captured (despite lack of anatomical clues) which conceived some protection to the reproductive tract; that Gelugob never cycled regularly and consequently developed less pathology; or that she never developed severe pathology in spite of cycling correctly and not breeding (less likely due to current scientific evidence).

4. Reproductive procedures conducted in female rhinoceroses

Different hormonal protocols including $PGF_{2\alpha}$, eCG, hCG, eFSH, hFSH/LH, altrenogest and CMA, were employed in the females under study with distinct purposes. In Gelugob, stimulation of follicular growth was not achieved using eCG, hFSH/LH, eFSH and hCG. At least eCG, however, resulted in increased blood flow to target areas and corresponding behavioural changes. As the follicular stock seemed depleted, Gelugob was declared to have reached early senescence at the approximate age of 30 years. In Puntung, $PGF_{2\alpha}$ was employed to promote luteolysis. Progesterone concentrations in mares reach basal levels within 24h of $PGF_{2\alpha}$ administration, and structural reduction is seen in the following 3 days (Miro, 2012). In Puntung's case, luteal structures were only slightly affected and progesterone was higher 3 days later (although it could have gone down first, unnoticed). Possible reasons for failure include a refractory period of the CL. Increased sampling for progesterone would be recommended to avoid pitfalls. Also in Puntung, hCG was employed to induce final maturation and ovulation in 24 to 48h. On both occasions the dominant follicle was relatively stable while the subordinate follicle was negatively affected and started regressing. Ovulation was not noted. Finally, both Puntung and Iman were subjected to long estrous synchronization protocols using synthetic progestins, which are supposed to suppress follicular development for a period longer than natural duration of the CL, and then potentiate follicular growth with a strong endogenous peak of FSH (Hermes *et al.*, 2012). Progestin downregulation treatments failed in both females, as noted by the development of follicles during suppression and low number of follicles at the end, although at least in Puntung CMA was able to inhibit ovulation and/or luteinization. Two hypotheses are considered: either length of the treatment was overcome by an active CL which later inhibited the FSH peak, or progesterone does not suppress FSH/LH release in this species (which may agree with previous findings of progesterone secretion and follicular development). In regard to altrenogest, Hermes and Hildebrandt (2012) reported a similar lack of success in other rhinoceros species due to a presumable lack of receptor affinity. Since hormonal protocols which are successful in other rhinoceros species seem to be ineffective in the Sumatran rhinoceros (or at least the two females under study), the author proposes the development of an hormonal treatment which mimics the apparent normal cycle and potentiates key points. The recommendation is that after ovulation/luteinization with GnRH, CMA would be administered in a lower dose at first, and then a higher dose in combination with eFSH, so that follicle growth is stimulated but ovulation or luteinization is inhibited. At the end of this protocol $PGF_{2\alpha}$ would be administered to induce luteolysis of any possible luteal tissue, and to allow for final growth in the next 24 to 48h (fig. 53). This would account for both hypotheses of CLs remaining active over one cycle and dominant follicles developing under the influence of progesterone.

Figure 53: Schematics of the proposed hormonal protocol using gonadotropin releasing hormone (GnRH), chlormadinone acetate (CMA), equine follicle stimulating hormone (eFSH) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).



Moving on to the analysis of conducted procedures, four different techniques were employed in Puntung in an attempt to remove endometrial cysts. While uterine lavage with povidone-iodine solution resulted in the aggravation of the cystic hyperplasia and disruption of estrous cyclicity, uterine flushing with cell culture medium apparently led to a significant reduction in extent and dimension of the cysts. Long term effects were not investigated and the treatment course was not pursued due to the extent of pathological alterations in Puntung's uterus. Two other techniques were employed in a direct approach to individual cysts, showing immediate results but eventual reappearance of the structures. Endoscopic-assisted laser photoablation as conducted in mares (Griffin & Bennett, 2002) was extremely complicated to employ due to the difficulty in overcoming the convoluted cervix, and reduced visibility from lack of insufflation and presence of fluids once the procedure had started. Consequently, the best approach for single large cysts seems to be direct aspiration, although previous dimensions were achieved in under 3 months. In this occasion transrectal access was established for the collection of oocytes, but aspiration could presumably also be conducted transcervically with ultrasonographic guidance and a specialized catheter.

As in both mares and rhinoceroses conception and full term gestation has been reported in the presence of single or small endometrial cysts (Griffin & Bennett, 2002; Roth, 2006) artificial insemination was attempted in Puntung after aspiration of the large cysts at the cranial ends of the uterine horns. The procedure was conducted as predicted but fertilization was not achieved most likely due to poor condition of the uterus and disrupted estrous cyclicity. No further attempts were conducted due to the extent of pathology in Puntung's uterus.

Regarding Iman, no attempts at improving uterine health were conducted due to the dimensions and structure of the tumours, difficulty in access and lack of technical options to apply in this situation. Iman's hydrosalpinx was however aspirated due to suspicions of it being a source of pain and discomfort. In mares, the hydrosalpinx is a rare condition which results from abnormal embryonic development or previous salpingitis (Larson, 2013). Treatment options were not found in the literature.

In regard to oocyte collection and *in vitro* techniques, several complications came up during the study period namely problems with the equipment and its adaptation to smaller animals, inability to maturate the oocytes and lack of good semen samples. In regard to necessary equipment, constant improvements were undertaken and an expert in the technique was consulted and invited to help. Follicle aspiration via transrectal access had a success rate of 66,7% to 100% in both Puntung and Iman (aspirated follicles vs identified follicles), while transvaginal aspiration was successful on 37,5% and 100% of follicles in Iman. Post-surgical complications were not noted in both females. Recovery of COCs was not achieved in Puntung during the study period, but was high in Iman with 66,7% to 75% of success (recovered COCs vs aspirated follicles). A total of 8 COCs was harvested of which 5 were reported to have reached metaphase II, revealing an apparent success rate of 62,5% for IVM. ICSI was performed in 3 oocytes but cleavage was not achieved. One hypothesis considered by the team was that observed "polar bodies" were in fact a result of fragmentation, which is related to poor quality or degenerating oocytes. Therefore, improvements in IVM of Sumatran rhinoceros oocytes seem necessary for the success of the technique.

5. Ultrasonographic findings in male rhinoceroses

The present study reports the anatomical structure of the urethra for the first time in the Sumatran rhinoceros. The description is in agreement with previous reports for other rhinoceros species, namely the presence of caudal and cranial urethral constrictions, the bulbous expansion of the pelvic urethra, and the *colliculus seminalis* (Forbes, 1880; Schaffer *et al.*, 1998; Schaffer *et al.*, 2001a). Measurements of the expanded pelvic urethra were similar to previously published values for other species (Schaffer *et al.*, 1998; Schaffer *et al.*, 2001a). In regard to the prostatic urethra, measurements were reported for the first time. Knowledge on the accessory sex glands of the Sumatran rhinoceros is clouded by uncertainty, with anatomical descriptions and measurements being neglected in the literature. Different characteristics were seen in the accessory sex glands of each male under study. Distinct ultrasonographic views used in each time frame account for some of the differences, namely in shape. The hyperechoic appearance of Tanjung's seminal glands is also believed to be a consequence of probe position, with the ultrasound waves going through fluid (the bladder as seen in fig. 44) and giving origin to an artefact. One other hypothesis to justify the differences is that Tanjung's glands were more active than Tam's, as suggested by a very large central vessel and thick surrounding muscular layer in the bulbourethral glands, larger dimensions for the prostate, and reports of strong libido and reproductive activity. In regard to measurements, the bulbourethral glands fell bellow the only reference for the species (Forbes, 1880) and measurements for the prostate were described for the first time. In what concerns the seminal glands, measurements revealed that Tanjung's were somewhat smaller than Tam's, and both were larger than reported by Zahari (1995) and much smaller than the reference from Forbes (1880). Interestingly, both the bulbourethrals and the prostate of Tam seem to have increased in size between 2009 and 2012 followed by a decrease in 2013 and 2014, while the seminal glands seem to have increased until 2014 and then decreased in 2015. Similar improvements until 2012 were also noted in semen quality and are likely to be associated with Gelugob being separated and later with the arrival of Puntung. The testicles respected previous descriptions for the species (Schaffer *et al.*, 1994;

Zahari, 1995), except for the presence of multiple *foci* of fibrosis. Testicular fibrosis is a common finding in male rhinoceroses over the age of 15, is correlated to age and is therefore regarded as a sign of testicular ageing (Hermes *et al.*, 2005). Testicular measurements fell below reference values, as did obtained measurements for the *cauda epididymidis* (Schaffer *et al.*, 1994; Zahari, 1995). Anechoic areas of fluid accumulation were seen surrounding the testicles and the epididymides of both males, with deposits reaching 1,5 by 3,0 cm below the *cauda epididymidis*. Going through the literature it is easy to find various references of free fluid surrounding testicles and epididymides of Sumatran rhinoceroses (Schaffer *et al.*, 1994; Zahari, 1995; Hermes *et al.*, 2006b). Some of the authors refer that fluid is mostly accumulated around the head of the epididymis, and not the tail as described in the present study (Schaffer *et al.*, 1994; Zahari, 1995). Upon observation of pictures of the posterior of Sumatran rhinoceros males (fig. 54), it becomes clear that confusion between descriptions of different authors may be due to individual differences between animals and between occasions for the same animal. While Tanjung exhibited what would in fact be described as a pendulous scrotum, Tam's testicles seemed to be much closer to the inguinal canal. This means that most likely testicles can acquire different positions and presumably fluid could be found surrounding the tail or the head of the epididymis. In what concerns the origin of the fluid, since vaginal and peritoneal cavities communicate through the inguinal canal, fluid found around testicles and epididymides may actually originate from the peritoneum. Intrinsic causes can be trauma, inflammation or tumours of intra-scrotal structures (no compatible observations), or simply impaired circulation (Schumacher & Varner, 2011). Some reports seem to associate high temperatures with hydrocele formation in horses due to maximum descent of the testicles compromising venous circulation in the spermatic cord. Although reports are ambiguous, hydrocele could depress or inhibit spermatogenesis due to increased temperature and/or pressure to vital structures (Schumacher & Varner, 2011). Consideration should be given to diagnostic and treatment of hydrocele in Tam to confirm if it affects semen quality.

Figure 54: Detail of the posterior of two male Sumatran rhinoceroses (Tam on the left, Tanjung on the right) for the comparison of testicular position. Copyright BORA.

6. Reproductive procedures conducted in male rhinoceroses

Semen collections in Tam went according to plan, following the advanced combined technique which has allowed for the best results in all rhinoceros species (Hermes & Hildebrandt, 2012; Hermes *et al.*, 2005). Obtained semen samples revealed values close to previous reports for Sumatran rhinoceroses (Zahari, 1995; Agil *et al.*, 2004; Agil *et al.*, 2008; O'Brien & Roth, 2000; Kretzschmar *et al.*, 2007; Hermes & Hildebrandt, 2012) but far away from references for other species using the same method (Hermes & Hildebrandt, 2012; Hermes *et al.*, 2009a; Hermes *et al.*, 2005; Hermes *et al.*, 2001a; Roth *et al.*, 2005; Reid *et al.*, 2009; Schaffer *et al.*, 1998). In regard to the timeline it is interesting to see that in 2011 Tam's semen showed the lowest parameters, most likely due to the coexistence with Gelugob. Semen quality started improving once Gelugob was moved to a separate facility, and in 2012 reached maximum values most likely motivated by the arrival of Puntung. No improvements were again seen for some time until 2014 and 2015 with some increases in concentration and volume. In conclusion, semen production of Tam and other male Sumatran rhinoceroses may be affected by several factors such as lack of reproductively active females and possibly males in the surrounding area, stress from living in close proximity to other rhinoceroses rather than solitarily, or simply stress from living in captivity (less likely due to good acceptance). In regard to preservation, Tam's semen showed a high sensitivity to chilling unlike what was previously reported for other rhinoceros species (Hermes & Hildebrandt, 2012; Hermes *et al.*, 2005; Hermes *et al.*, 2001a). Post-thawing motility was reported below 5% as expected from the low quality of Tam's semen samples. Directional freezing would therefore be a valuable help to Tam's semen samples if the logistics of using vulnerable electronics in a jungle setting can be worked out. Concerning Tanjung, although he was reported to have high libido yet poor semen, data from collections was not available for the period under study. As a last note, it was overwhelming to find that twice all semen samples from Tam were lost due to human error. High priority should be given to the preservation of samples as important as these, and extreme caution should be demanded from those in charge of the task. Consideration should be given to separating samples between different institutions as a way to safeguard them from adversities, although transportation of cryopreserved material can be challenging.

7. Other findings

Examination of the urinary bladder in both females and males revealed the presence of large amounts of urinary sediment. This finding is reported as common in other rhinoceros species (Hermes, personal communication; Haffey, Pairan, Reinhart & Stoops, 2008) and as in horses, is due to large amounts of mucus and calcium carbonate crystals in the urine. Several authors claim that alkaline urine predisposes to crystal formation in horses and rhinoceroses (van Metre, 2009; Haffey *et al.*, 2008). Clauss *et al.* (2007) further explains that most hindgut-fermenting herbivores absorb more calcium than necessary to meet their requirements, and consequently excrete the excess in urine. Furthermore, in species adapted to diets with higher calcium/phosphate ratios such as the Black rhinoceros (and presumably also the Sumatran rhinoceros), calcium absorption is even more efficient (Clauss *et al.,* 2007). At the BRS the animals are fed a selection of plants collected daily from the forest in resemblance to their natural diets as described by previous ecological studies in Sabah. Hence the probability of this being a consequence of incorrect feeding and nutrition in captivity is slimmer than expected.

Another finding in all female rhinoceroses was excessive peritoneal fluid. Although males were not affected by accumulations of abdominal fluid, both exhibited hydroceles which could be related to this condition in the females. Excessive peritoneal fluid is commonly associated to several pathological alterations of the abdominal organs in the horse. Generally, any condition which causes changes in plasma hydrodynamic or oncotic pressure, or inflammation of infectious or non-infectious origin, can produce excessive peritoneal fluid (Swanwick & Wilkinson, 1976). Apart from reproductive pathology, the females under study did not show any clinical signs which gave meaning to this finding. The hypothesis of this being associated to diet or other common factor between several Sumatran rhinoceroses remains unstudied.

CONCLUSION

It is the author's belief that the present study achieved the aims to which it aspired. This dissertation constitutes the most extensive and thorough report of ultrasonographic findings in the Sumatran rhinoceros, and contributes with new technical information on numerous anatomical structures such as the ovarian luteal structures, the male urethra and the male accessory sex glands. The present report also initiates the discussion on various important topics in Sumatran rhinoceros reproduction such as ovarian dynamics, estrous cyclicity, low quality of semen samples and hydrocele development in male individuals; and puts forward several hypotheses for future consideration and investigation. Notably, strong evidence to contradict the long standing belief of Sumatran rhinoceroses exhibiting induced ovulation is also presented. Several reproductive procedures were described as conducted in the Sumatran rhinoceroses under study, most of them for the first time in the species. Although so far most procedures failed in achieving their purpose, incredibly valuable information can be extracted from their analysis as discussed in the present report. Here it is important to mention that in spite of numerous and repeated interventions, challenging and invasive procedures, and often lack of optimal health, none of the animals under study suffered from negative consequences directly or indirectly attributable to the interventions, which notes an extremely careful and responsible management of the animals and their valuable lives.

As a last point, the distressing discovery of a phenomenon related to lack of breeding in captive rhinoceroses, occurring naturally in the wild finally explains why wild populations have been continuously declining over decades even when poaching is under control and habitat is available. So as it turns out, for the last decades the low density in itself has been the major threat to Sumatran rhinoceros survival, meaning that protection in the wild is no longer enough and that Indonesia (as the last country to hold wild populations) must take active measures to ensure the survival of the species. Furthermore, collaboration between governments, relevant international associations, non-governmental organizations and research institutions is essential to optimize the efforts of Sumatran rhinoceros conservation. As long as there is hope and will to collaborate on an international level, the specialized reproduction management team from the IZW will continue working with the Borneo Rhino Alliance with the purpose of creating embryos from these that may be the last rhinoceroses of Borneo.

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APPENDIX: ABSTRACTS

1. International Conference on Diseases of Zoo and Wild Animals 2015, Barcelona

Proc Int Conf Dis Zoo Wild Anim 2015 *Short abstract*

THE SABAH RHINO (*DICERORHINUS SUMATRENSIS HARRISSONI*) BREEDING PROGRAMME: CURRENT SITUATION AND FIRST CONCLUSIONS

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The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is on the verge of extinction. Once found throughout Southeast Asia, it stands now with less than 100 individuals scattered mainly in three national parks in Sumatra. In Sabah (Malaysian Borneo), as well as in Peninsular Malaysia, the Sumatran rhinoceros is now considered to be "functionally extinct". Between 1984 and 2014 forty-five Sumatran rhinoceroses were captured from the wild, resulting in only two successful breeding pairs. In early 2015 only nine individuals survive in captivity: three (1.2) in the Borneo Rhino Sanctuary, in Sabah; five (2.3) in the Sumatran Rhino Sanctuary, in Sumatra; and only one (1.0) left in Cincinnati Zoo, USA. Since 2009 the Leibniz Institute for Zoo and Wildlife Research (IZW) has been collaborating with the Borneo Rhino Alliance (BORA), mainly through the use of advanced imaging and assisted reproduction technologies on the 1.3 wild-caught Bornean rhinoceroses (*Dicerorhinus sumatrensis harrissoni*) held at the Borneo Rhino Sanctuary. Throughout fourteen visits to Sabah, the IZW team and associated experts had the chance to perform forty-two reproductive assessments, six semen collections by electroejaculation, three endometrial cyst removal procedures, one artificial insemination (AI), three oocyte collections ("ovum pick-up", OPU) and one intracytoplasmic sperm injection (ICSI). In what concerns the male, significant improvements in semen quality were observed throughout time: despite being considered reproductively inactive in 2009 and 2011, in 2014 a maximum concentration of 5×10^6 sperm cells /mL was obtained, with 65 % motility. When captured from the wild, two cycling females showed severe reproductive pathology that rendered them incapable of carrying a pregnancy, namely extensive cystic endometrial hyperplasia and a large number of uterine leiomyomas. Four different techniques where used to remove endometrial cysts: uterine lavage with cell medium M199 (Sigma-Aldrich Chemie GmbH, Munich, Germany) and povidone-iodine solution (Braunol®, B. Braun Melsungen AG, Germany), endoscopic assisted laser photoablation and ultrasound guided aspiration. These procedures proved to be of very limited success. Consequently, AI was attempted only once and no fertilisation occurred due to poor condition of the uterus and irregular cycling. As natural conception was excluded, *in vitro* fertilisation was attempted for the first time in Sumatran rhinoceros. OPU was performed on three occasions and successful on two, with the total collection of five oocytes. ICSI was performed with two of the oocytes, but cleavage was not achieved. Furthermore, to preserve the genome of these that may well be the last Bornean rhinoceroses, cell cultures were established from skin and mucosal samples and cryopreserved until seven passages, for future development of induced pluripotent stem cells.

2. 10th International Conference on Behaviour, Physiology and Genetics of Wildlife, Berlin 2015

The Sabah Rhino Breeding Programme: reproductive pathology and its clinical implications in wild-caught Sumatran rhinoceroses

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Keywords: Sumatran, rhinoceros, reproductive pathology, cyst, leiomyoma

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) is on the verge of extinction. Intensive hunting and habitat loss contributed to a precipitous decline in its numbers during the first half of the 20th century. In 1984 it was agreed that in addition to the protection of wild individuals, it was time to establish a captive breeding programme. By 2014, 39 of the original 40 wild-caught animals were dead, and a species that was once found throughout Southeast Asia was reduced to less than 100 individuals scattered mainly in three national parks in Sumatra. In the last decade poaching and habitat loss were no longer the main threats to the Sumatran rhinoceros. It is clear that very low densities of a slow-breeding solitary species like the Sumatran rhinoceros drastically decrease the possibility of finding a reproductively active partner in the short frame of sexual receptivity (one day of oestrus in a 21 to 27-day cycle), leading to long non-reproductive periods. As described for other rhinoceros species, the organs of non-breeding females are exposed to sex steroids for prolonged periods of time due to continuous ovarian cycle activity, which results in the development of reproductive pathology, depletion of the follicular stock and premature senescence. Several reports of reproductive pathology in Sumatran rhinoceros suggest that at least 50 % of wild-caught females were affected by this phenomenon known as “asymmetric reproductive aging”, with variable consequences to their fertility. Since 2009 the Leibniz Institute for Zoo and Wildlife Research (IZW) has been collaborating with the Borneo Rhino Alliance (BORA) through the use of advanced imaging and assisted reproduction techniques on the 1.3 Bornean rhinoceroses (*Dicerorhinus sumatrensis harrissoni*) held at the Borneo Rhino Sanctuary, in Sabah. When captured from the wild, both cycling females showed severe reproductive pathology that rendered them incapable of carrying a pregnancy, namely extensive cystic endometrial hyperplasia and a large number of uterine leiomyomas. Four different techniques were used for the removal of endometrial cysts: uterine lavage with cell medium M199 and povidone-iodine solution, endoscopic assisted photoablation and ultrasound-guided aspiration. These procedures proved to be of limited success. Consequently artificial insemination was attempted only once and no fertilisation occurred. As natural conception was excluded, the focus of the programme turned to *in vitro* fertilisation as a last resource for reproduction.

The Sabah Rhino Breeding Programme: reproductive pathology of wild-caught Sumatran rhinoceroses and its implications in conservation

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Keywords: rhinoceros, Sumatran, reproductive pathology, cyst, leiomyoma

The Sumatran rhinoceros (*Dicerorhinus sumatrensis*) was once found throughout Southeast Asia, but intensive hunting and habitat loss contributed to a precipitous decline in its numbers during the first half of the 20th century. In 1984 it was agreed that in addition to the protection of wild individuals it was time to establish a captive breeding programme. In the 32 years that followed, 46 animals were captured from the wild, resulting in only 2 breeding pairs and the death of 41 individuals. The Sumatran rhinoceros stands now on the verge of extinction, with only 9 captive animals and less than 100 free-roaming individuals scattered in three national parks in Sumatra. Since 2009 the Leibniz Institute for Zoo and Wildlife Research (IZW) has been collaborating with the Borneo Rhino Alliance (BORA) through the use of advanced imaging and assisted reproduction techniques on the 1.3 Bornean rhinoceroses (*Dicerorhinus sumatrensis harrissoni*) held at the Borneo Rhino Sanctuary, in Sabah. When captured from the wild, both young cycling females showed severe reproductive pathology that rendered them incapable of carrying a pregnancy, namely extensive cystic endometrial hyperplasia and a large number of uterine leiomyomas. Going back in time, several reports of reproductive pathology in Sumatran rhinoceroses suggest that at least 50% of wild-caught females were affected by similar conditions, with variable consequences to their fertility. It becomes clear that low densities of a slow-breeding solitary species like the Sumatran rhinoceros drastically decrease the possibility of finding a reproductively active partner in the short frame of sexual receptivity (one day of oestrus in a 21 to 27-day cycle), thus leading to long non-reproductive periods in wild individuals. As described for other rhinoceros' species in captivity, the organs of non-breeding females are exposed to sex steroids for prolonged periods of time due to continuous ovarian cycle activity. This results in a phenomenon known as "asymmetric reproductive aging", which comprises the development of reproductive pathology, depletion of the follicular stock and premature senescence. The distressing discovery of this phenomenon occurring naturally in free-ranging Sumatran rhinoceroses clearly reflects the "Allee effect", which postulates that with low population size or density comes reduced individual fitness. The "Allee effect" and more specifically the phenomenon of "asymmetric reproductive aging" are now believed to play, both in the past and at present time, one of the most important roles in the fall of the Sumatran rhinoceros.